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AUTHOR(S):

Nanba, Hirokazu

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New Biotransformation Process for the Production of D-Amino Acids

Hirokazu Nanba

1999

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PUBLICATIONS

ABBREVIATIONS

DCase	*N*-Carbamyl-D-amino acid amidohydrolase
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate
DEAE-	Diethylaminoethyl-
E. coli	*Escherichia coli*
Tris	Tris(hydroxymethyl)aminomethane
HPLC	High-performance liquid chromatography
LB	Luria-Bertani's broth
PCR	Polymerase chain reaction
Km	Michaelis constant
Ki	Inhibitor constant

INTRODUCTION

This thesis describes studies on a new process for the production of D-amino acids involving enzymatic reactions.

Enzymatic transformation is a superior method because of its high selectivity and high efficiency under mild conditions. Therefore, starting with the transformation and synthesis of steroids, it has been used for the production of various useful chemicals. Among them, D-amino acids are intermediates useful in the preparation of physiologically active peptides, and β -lactam antibiotics such as semisynthetic penicillins and cephalosporins. For example, D-*p*-hydroxyphenylglycine is the side chain of amoxicillin (Fig. 1), which is one of the most popular antibiotics in the world. Several procedures for the chemical[1)] and enzymatic[2-19)] production of optically active amino acids have been devised, some of which have been used commercially. Among them, as shown in Fig. 1, a method involving D-specific hydantoinase (EC 3.5.2.2.), which catalyzes the first step, i. e. reversible ring opening through the hydrolysis of DL-5-substituted hydantoin to *N*-carbamyl-D-amino acids, is of special interest. With the spontaneous racemization of 5-substituted hydantoin during the reaction, the racemic substrate is converted quantitatively to *N*-carbamyl-D-amino acids. The *N*-carbamyl-D-amino acids produced are transformed chemically or enzymatically to the corresponding D-amino acids with retention of their original configurations.

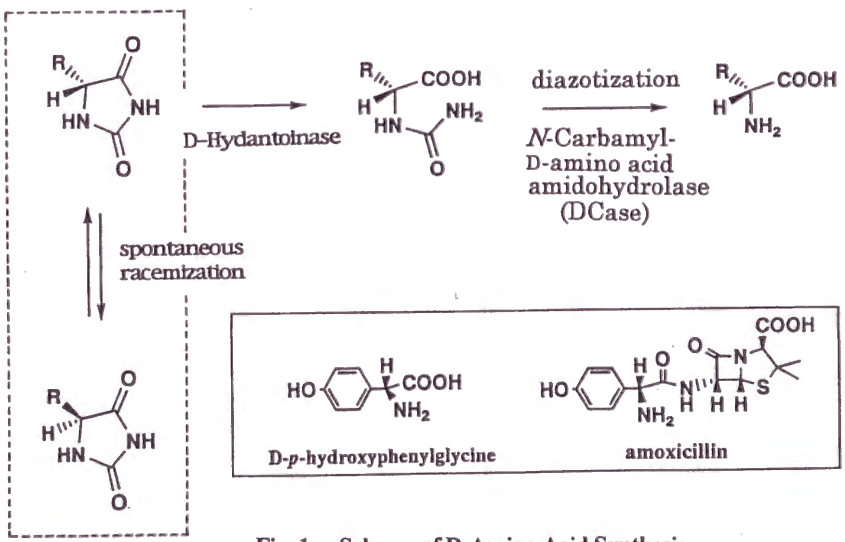

Fig. 1. Scheme of D-Amino Acid Synthesis.

N-Carbamyl-D-amino acid amidohydrolase (DCase) catalyzes the second step in Fig. 1, involving the hydrolysis of *N*-carbamyl-D-amino acids to D-amino acids, ammonia and carbon dioxide. If this enzyme is immobilized as a hydantoinase, which has been used as an immobilized enzyme practically,[20] that is to say, the process comprises a bioreactor, D-amino acid production will be improved. Immobilized enzymes have been used commercially for production processes in various fields, such as the food and pharmaceutical industries.[21-23] There are great advantages in not only that the enzymes can be reused for the reaction, but also that the products can be refined easily, the yields can be improved, and the process can be simplified. Because of the industrial importance of DCase, there have been several reports on this enzyme of *Pseudomonas*,[6] *Agrobacterium*,[10,11,13,16-19] *Arthrobacter*,[12] *Blastobacter*,[14] and *Comamonas*.[15] Some have reported the properties of the enzyme. But only Ogawa *et al.* have investigated purified enzymes in detail, i. e. those of *Blastobacter*[14] and *Comamonas*.[15] Studies on the cloning and expression of the enzyme have been only reported for an *Agrobacterium* species.[18,19] Regarding immobilization, only Olivieri *et al.*[11] have reported that they immobilized whole cells of *Agrobacterium*, but the details have not been given. As mentioned above, immobilization is advantageous, so I tried to immobilize DCase and to apply it to the production of D-amino acids practically. To achieve this, highly active DCase was obtained from a natural source, its thermostability was improved by gene mutation,[36,37] it was overexpressed in *Escherichia coli* using recombinant gene technology, and it was immobilized by a method with which it is stable during a long term reaction. Furthermore, I studied the properties of the enzyme for practical reasons and also to facilitate understanding of its physiologic functions.

Chapter Ⅰ describes the screening for microorganisms producing DCase, cloning of the DCase gene, and overexpression and some properties of the enzyme. Chapter Ⅱ describes the immobilization of DCase obtained in Chapter Ⅰ, its stabilization, and its use for repeated batch reactions. The stability of the immobilized DCases of thermotolerant bacteria is also described. Chapter Ⅲ describes the effect of the thermostability of the improved DCase[36,37] on the stability of the immobilized enzyme in repeated batch reactions,

and the characteristics of the immobilized enzyme. Chapter IV describes the construction of an expression plasmid, and economical hyperproduction of the DCase in *E. coli*.

CHAPTER I

Isolation of *Agrobacterium* sp. Strain KNK712 That Produces *N*-Carbamyl-D-Amino Acid Amidohydrolase, Cloning of the Gene for this Enzyme, and Properties of the Enzyme

As mentioned in the introduction to this thesis, D-amino acids are important intermediates for β-lactam antibiotics and physiologically active peptides. These optically active amino acids can be produced efficiently with a 2-step reaction involving hydantoinase and DCase, which catalyzes the hydrolysis of *N*-carbamyl-D-amino acids to D-amino acids, ammonia and carbon dioxide.

Several microorganisms producing both D-stereospecific DCase and D-hydantoinase have been isolated: *Pseudomonas*,[6] *Agrobacterium*,[10, 11, 13, 16-19] *Arthrobacter*,[12] *Blastobacter*,[14] and *Comamonas*[15] (which only produces DCase). Louwrier and Knowles have reported an *Agrobacterium* sp.[16, 17] that produces DCase, but they did not mention whether or not D-hydantoinase is also produced. Both enzymes are suitable for use in the production of D-amino acids.

DCase has been studied by several groups,[6, 10-19] but few studies on the cloning and expression of the DCase gene, which would be useful in the production of D-amino acids, have been reported.[18, 19] Some properties of the enzyme have been reported.[6, 10-19] More information about DCase is needed for both practical reasons and also for understanding its physiologic functions. To make the production of D-amino acids more efficient, and to understand DCase further, I set out to overexpress DCase in *E. coli*, and to characterize the enzyme. In this chapter, I describe the screening for microorganisms producing DCase, cloning of the DCase gene, and overexpression and some properties of the enzyme.

MATERIALS AND METHODS

Media and culture conditions. Medium A: A mixture of 10 g of glycerol, 5 g of glucose, 3.5 g of KH_2PO_4, 3.5 g of Na_2HPO_4, 0.5 g of $MgSO_4 \cdot H_2O$, 20 mg of $MnCl_2 \cdot 4H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, and 3 g of a nitrogen source, *N*-carbamyl-D-*p*-hydroxyphenylglycine or *N*-carbamyl-D-phenylglycine, in 1 liter of tap water, pH 7.0, was used for screening for bacteria with DCase activity. Medium B: A mixture of 10 g of meat extract, 10 g of peptone, 5 g of yeast extract, and 20 g of agar in 1 liter of tap water, pH 7.0, was used for the isolation of microorganisms. Medium C: A mixture of 25 g of glycerol, 5 g of glucose, 7 g of KH_2PO_4, 7 g of Na_2HPO_4, 1 g of $MgSO_4 \cdot H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, 2 g of urea, 4 g of yeast extract, and 1 g of *N*-carbamyl-D-*p*-hydroxyphenylglycine in 1 liter of tap water, pH 6.5, was used at 33℃ for the growth of cells of *Agrobacterium* sp. strain KNK712, used for the hydantoinase and DCase reactions.

E. coli JM109[24] carrying recombinant plasmids was grown in Luria-Bertani[25] medium containing 100 mg of ampicillin and 1 mmol of isopropyl- β -D-thiogalactopyranoside per liter at 37℃. M9 medium[25] containing 1 g of 5-methylhydantoin as the sole nitrogen source (instead of NH_4Cl), 50 mg of ampicillin and 1 mg of thiamine, in 1 liter (medium D) was used for cloning of the DCase gene.

Chemicals. 5-Substituted hydantoins were prepared from the corresponding aldehydes[26] or amino acids.[27] *N*-Carbamylamino acids were prepared from the corresponding amino acids.[28]

The other chemicals used in this work were the best available commercial products.

Screening methods. For the isolation of microorganisms that can hydrolyze *N*-carbamyl-D-*p*-hydroxyphenylglycine to D-*p*-hydroxyphenylglycine, a spoonful of soil was suspended in 10 ml of saline, and then 300 μl of the supernatant was used to inoculate 10 ml of medium A in a test tube. After aerobic cultivation at 30℃ until growth was detected

visually, reduction of *N*-carbamylamino acids and production of amino acids were detected by thin-layer chromatography. Microorganisms from culture broths in which *N*-carbamylamino acids disappeared or amino acids accumulated, were isolated on agar plates of medium B.

Gene cloning. Chromosomal DNA was prepared from *Agrobacterium* sp. strain KNK712 by the method of Marmur[29] and partially digested with *Sau*3A I. Then DNA fragments 4 to 9 kbp long were fractionated by agarose gel electrophoresis. The DNA fragments were ligated into the *Bam*H I site of pUC18, and then used to transfect cells of *E. coli* JM109. Recombinant colonies that grew on the agar plates were collected, suspended in saline, and used to inoculate 10 ml of medium D in test tubes, which were cultured aerobically at 37℃. Culture broth in which growth was detected visually was transferred to the same medium and culture was continued. After this enrichment culture was repeated a total of three times, the recombinants with the DCase gene were isolated on agar plates of LB medium containing 100 mg of ampicillin per liter. From one of the isolated strains, a plasmid harboring the DCase gene was prepared by the alkaline lysis.[30]

DNA sequencing. The 1.8-kp *Eco*R I -*Hind*Ⅲ fragments of pAD108 were digested with appropriate restriction endonucleases and then cloned in both orientations into M13mp18 and M13mp19. The resultant recombinants were used to transfect *E. coli* cells so that single-stranded templates were produced. The nucleotides of the clones were sequenced by the dideoxy chain termination method[31] with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio).

Identification of DCase. *E. coli* JM109 carrying pAD108 was grown in 10 ml of LB medium containing 100 mg of ampicillin and 1 mmol of isopropyl- β -D-thiogalactopyranoside per liter at 37℃ for 20 h. The cells were harvested by centrifugation (3000 × g, 5 min, 4℃), suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.0),

and disrupted by sonication. The cell debris was removed by centrifugation (18,000 × g, 10 min, 4℃). The supernatant was heated in sample buffer containing 5% β-mercaptoethanol and 2% SDS for 5 min over boiling water, and then analyzed on a 10% polyacrylamide gel by the method of Laemmli.[32] After electrophoresis, the gel was stained with Coomassie brilliant blue. For measurement of the staining at 550 nm, a dual-wavelength flying-spot scanner CS-9000 (Shimadzu, Kyoto, Japan) was used.

Purification of DCase. All procedures were done at 4℃. *E. coli* JM109 cells carrying pAD108 from 9.5 liters of culture broth were suspended in 20 mM Tris-HCl, pH 7.0, containing 5 mM dithiothreitol (DTT) and 50 μM camostat mesilate (Foipan, Ono Pharmaceutical Co., Osaka, Japan), and the suspension was brought to 475 ml and disrupted with ultrasound. The cell debris was removed by centrifugation. The resulting supernatant was heated at 50℃ for 30 min. After centrifugation, the supernatant obtained, (460 ml), was put on a phenyl-Sepharose column (2.5 × 38 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM DTT, 50 μM camostat mesilate, 1 M NaCl, and 0.3 M ammonium sulfate, and eluted with 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT and 50 μM camostat mesilate. The fractions containing enzyme activity were combined (700 ml).

The enzyme solution was fractionated with solid ammonium sulfate. The precipitate obtained at 0-30% saturation was collected by centrifugation, dissolved in 600 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM DTT, and dialyzed.

The enzyme solution was put on a DEAE-Sepharose column (2.5 × 18 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM DTT, and eluted with 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM DTT. The active fractions were combined (105 ml) and concentrated to 18 ml with an membrane filter apparatus (Amicon Corp., Lexington, Mass.) equipped with a YM-10 membrane. The enzyme solution was dialyzed twice against 2 liters of 10 mM sodium phosphate buffer, pH 7.15, containing 0.15 M NaCl.

DCase assay. DCase activity was assayed by measurement of D-*p*-hydroxyphenylglycine produced from *N*-carbamyl-D-*p*-hydroxyphenylglycine. The reaction was started by the addition of 100 μl of enzyme solution diluted with 100 mM potassium phosphate buffer, pH 7.0, containing 5 mM DTT to the assay mixture containing 47.6 μmol of *N*-carbamyl-D-*p*-hydroxyphenylglycine and 100 μmol of potassium phosphate buffer, pH 7.0, in a total volume of 1 ml. After 20 min of incubation at 40℃, the reaction was stopped by the addition of 250 μl of 20% trichloroacetic acid. The D-*p*-hydroxyphenylglycine produced was analyzed by high-pressure liquid chromatography. One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of D-*p*-hydroxyphenylglycine at the rate of 1 μmol·min^{-1} under the assay conditions used.

Analytical methods. Qualitative analysis of *N*-carbamylamino acids and amino acids was done by silica-gel thin-layer chromatography with a solvent system of *n*-butanol-acetic acid-water (4:1:1, by vol.). The compounds were detected with *p*-dimethylaminobenzaldehyde-6 N HCl and ninhydrin, respectively.

N-Carbamylamino acids and amino acids were measured by high-pressure liquid chromatography (Shimadzu LC-6A) at 210 nm on a 25-cm Finepack SILC18-5 column (Jasco, Tokyo, Japan) at the flow rate of 1 ml·min^{-1} with 36.7 mM KH_2PO_4 containing 15% methanol adjusted to pH 2.5 with phosphoric acid.

The steric configurations of *N*-carbamyl-*p*-hydroxyphenylglycine and *p*-hydroxyphenylglycine were identified by measurement of specific optical rotation.

Ammonia was assayed by the indophenol method.[33]

For the measurement of D-amino acids with D-amino-acid oxidase (EC 1.4.3.3), an assay mixture of 100 mM Tris-HCl, pH 8.0, 10.6 mM phenol, 0.51 mM 4-amino antipyrine, 1 U·ml^{-1} D-amino-acid oxidase from porcine kidney (Sigma), 0.71 U·ml^{-1} peroxidase from horseradish (Sigma), and 30 μl of sample was incubated for 1 h at 30℃, and the absorbance at 505 nm was measured.

Protein was assayed by the method of Bradford.[34]

RESULTS

Isolation of microorganisms that hydrolyze N-carbamyl-D-amino acid

Twenty-eight strains with DCase activity were isolated after enrichment culture with *N*-carbamyl-D-*p*-hydroxyphenylglycine or *N*-carbamyl-D-phenylglycine as the sole nitrogen source from a total of 1190 soil samples. Strain KNK712 had the highest DCase activity of these 28 strains, and was identified as an *Agrobacterium* sp.

With resting cells of *Agrobacterium* sp. strain KNK712, the hydantoinase and DCase reactions were carried out in potassium phosphate buffer. DL-5-(*p*-Hydroxyphenyl)hydantoin and *N*-carbamyl-DL-*p*-hydroxyphenylglycine were used as the substrates for the hydantoinase and DCase reactions, respectively. *N*-Carbamyl-*p*-hydroxyphenylglycine and *p*-hydroxyphenylglycine were produced from 5-(*p*-hydroxyphenyl)hydantoin and *N*-carbamyl-*p*-hydroxyphenylglycine, respectively, and were isolated by concentration, extraction, and crystallization. The crystals had about the same specific optical rotations of authentic *N*-carbamyl-*p*-hydroxyphenylglycine and D-*p*-hydroxyphenylglycine (Table 1). Strain KNK712 had both hydantoinase and DCase activity, and both enzymes had strict D-form stereoselectivity.

Table 1. Hydantoinase and DCase Reactions with KNK712.

Substrate, mmol	After reaction				
	HPG		C-HPG		HPGH
	mmol	$[\alpha]_D^{25}$	mmol	$[\alpha]_D^{25}$	mmol
DL-HPGH 26.0	3.4		16.0	-175.9	6.6
C-DL-HPG 23.8	11.8	-157.9	12.0	+175.9	

HPG, *p*-hydroxyphenylglycine; C-HPG, *N*-carbamyl-*p*-hydroxyphenylglycine; HPGH, 5-(*p*-hydroxyphenyl)hydantoin.

Reactions were at 41℃ for 19 h in a reaction mixture containing 5.0 g of DL-HPGH for the hydantoinase reaction, and 5.0 g of C-DL-HPG for the DCase reaction, 10 mmol of potassium phosphate buffer, and intact cells of *Agrobacterium* sp. strain KNK712 harvested from 100 ml of broth, in a total volume of 100 ml. During the reaction, the pH of the mixture was kept at 8.9 with 2.5 N NaOH for the hydantoinase reaction and at pH 6.9 with 2.5 N HCl for the DCase reaction. HPG and C-HPG were isolated, and their specific optical rotations were measured.

Authentic C-D-HPG $[\alpha]_D^{25}$ -174.7 (c 1, 1 N NaOH)

Authentic D-HPG $[\alpha]_D^{25}$ -158.3 (c 1, 1 N HCl)

Cloning of gene for DCase

Recombinant clones that can utilize 5-methyl-DL-hydantoin as a nitrogen source were isolated. One of the clones contained a plasmid carrying both the hydantoinase and DCase genes. This plasmid, designated pAD101 comprised an insert of a 4.7-kbp DNA fragment of *Agrobacterium* sp. strain KNK712 in vector plasmid pUC18. A restriction map of pAHD101 is shown in Fig. 2.

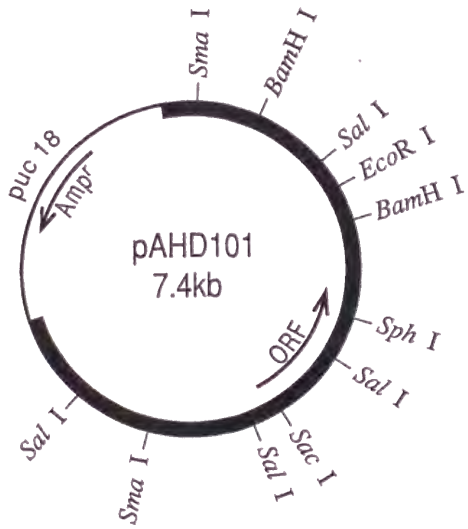

Fig. 2. Structure of pAD101.

The thick line represents the fragment from the chromosomal DNA of KNK712. The thin line represents the pUC18 vector. The open reading frame (ORF) of the DCase gene is indicated by one arrow, and the other arrow shows the Amp^r gene.

Plasmid pAD101 was digested with *Sma* I and *Eco*R I . A 2.7-kbp *Sma* I -*Eco*R I fragment was ligated with pUC19 digested with *Sma* I and *Eco*R I , giving plasmid pAD107. Plasmid pAD107 was partially digested with *Sal* I , and plasmid pAD108 was obtained by cyclizing of the 4.5-kbp DNA fragment containing pUC19. This plasmid contained a 1.8-kbp DNA fragment carrying the DCase gene.

Cell extracts of *E. coli* JM109 carrying pUC19 and *E. coli* JM109 carrying pAD108 were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). A band of 34 kDa arising from DCase was found. The staining was measured at 550 nm with a spot scanner, and results showed that DCase accounted for 50% of the total soluble protein of the cells. The DCase gene was overexpressed under the control of the *lac* promoter. The specific activity of the cell extract was 3.3 units per milligram of protein, about 10 times the specific activity of *Agrobacterium* sp. strain KNK712.

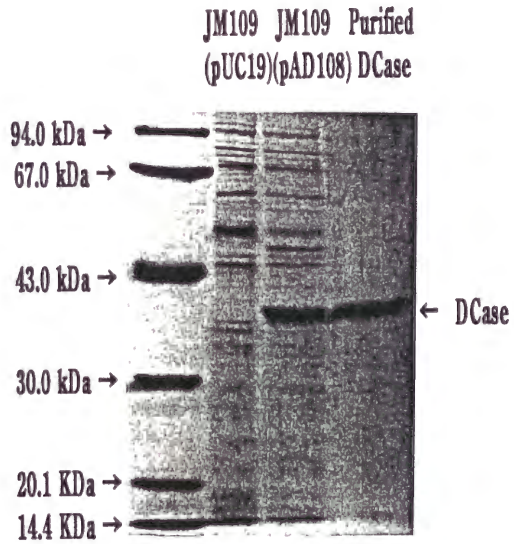

Fig. 3. Results of SDS-Polyacrylamide Gel Electrophoresis.

A cell extract of *E. coli* carrying either pUC19 or pAD108 was analyzed, as was purified DCase. The peptide band of the DCase is indicated by the arrow labelled "DCase". Molecular mass markers: phosphorylase b, 94.0 kDa; serum albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4kDa.

Purification of DCase

Before characterization, the DCase expressed in *E. coli* was purified. The enzyme was purified 3.9-fold with a yield of 12.3% and the specific activity was 7.20 units per milligram of protein. The results of SDS-polyacrylamide gel electrophoresis of the purified enzyme are shown in Fig. 3.

Nucleotide sequence

The sequence of the 1.8-kbp insert from pAD108 is shown in Fig. 4. One open reading frame was found, and it consisted of 912 bases with a starting triplet, ATG, at position 230 and an ending triplet, TGA, at position 1144. The open reading frame was predicted to encode a polypeptide of 304 amino acids, with a calculated molecular weight of 34,285. The deduced N-terminal amino acid sequence coincided with the sequence of the purified DCase from *Agrobacterium* sp. strain KNK712 (20 amino acids except for an N-terminal methionine; sequence not shown).

Properties of DCase

For characterization, the DCase expressed in *E. coli* was used. The optimum pH was about 7.0, and the optimum temperature was about 65℃ (Fig. 5). The enzyme was most stable at about pH 7.0, stable at 55℃ or less, and unstable at 60℃ or more (Fig. 6).

The effects of various reagents on the enzyme activity are shown in Table 2. The enzyme lost more or all of its activity with the SH reagents used and with Cu^{2+}, Hg^{2+}, and Ag^{+}.

The K_m values for *N*-carbamyl-D-*p*-hydroxyphenylglycine at pH 7.0 and 8.0 were 0.89 and 0.50 mM, respectively.

The enzyme was inhibited by ammonium chloride. The inhibition was of the mixed type, and the inhibitor constants at pH 7.0 were 32.6 mM for K_i and 101 mM for K_i', at pH 8.0, they were 7.50 mM for K_i and 13.3 mM for K_i'. The enzyme was inhibited more at pH 8.0 than at pH 7.0.

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GTCGACGGCGGGCTCGCGCGAGAGCTTGTCAAGCAGCGCAAATTCCGGTTCCGCTCCGGTTGACAGATCAAAAATTTTACGCCTGTTATTGTCGTGCTGC  100

ATGTAATATTTCGTACTTTATGTAGAATTTGCATTGCGCCGCGAGTCACAAAGCCGGTTTTCGGCGATGTGTTTCACAACGTTTTCCCGGCCGCTGGGCC  200

GGACATCACCTAGGAAGGAGCAGAGGTTCATGACACGTCAGATGATACTTGCAGTGGGACAACAAGGTCCGATCGCGCGCGCGGAGACACGCGAACAGGT  300
                              M  T  R  Q  M  I  L  A  V  G  Q  Q  G  P  I  A  R  A  E  T  R  E  Q  V

CGTCGTTCGTCTTCTCGACATGCTGACGAAAGCCGCGAGCCGGGGCGCGAATTTCATTGTCTTCCCCGAACTCGCGCTTACGACCTTCTTCCCGCGCTGG  400
 V  V  R  L  L  D  M  L  T  K  A  A  S  R  G  A  N  F  I  V  F  P  E  L  A  L  T  T  F  F  P  R  W

CATTTCACCGACGAGGCCGAGCTCGATAGCTTCTATGAGACCGAAATGCCCGGCCCGGTGGTCCGTCCACTCTTTGAGAAGGCCGCGGAACTCGGGATCG  500
H  F  T  D  E  A  E  L  D  S  F  Y  E  T  E  M  P  G  P  V  V  R  P  L  F  E  K  A  A  E  L  G  I  G

GCTTCAATCTGGGCTACGCTGAACTCGTCGTCGAAGGCGGCGTCAAGCGTCGCTTCAACACGTCCATTTTGGTGGATAAGTCAGGCAAGATCGTCGGCAA  600
  F  N  L  G  Y  A  E  L  V  V  E  G  G  V  K  R  R  F  N  T  S  I  L  V  D  K  S  G  K  I  V  G  K

GTATCGTAAGATCCATTTGCCGGGTCACAAGGAGTACGAGGCCTACCGGCCGTTCCAGCATCTTGAAAAGCGTTATTTCGAGCCGGGCGATCTCGGCTTC  700
 Y  R  K  I  H  L  P  G  H  K  E  Y  E  A  Y  R  P  F  Q  H  L  E  K  R  Y  F  E  P  G  D  L  G  F

CCGGTCTATGACGTCGACGCCGCGAAAATGGGGATGTTCATCTGCAACGATCGCCGCTGGCCTGAAGCCTGGCGGGTGATGGGCCTCAGGGGCGCCGAGA  800
P  V  Y  D  V  D  A  A  K  M  G  M  F  I  C  N  D  R  R  W  P  E  A  W  R  V  M  G  L  R  G  A  E  I

TCATCTGCGGCGGCTACAACACGCCGACCCACAATGCCCCTGTTCCCCAGCACGACCACCTGACGTCCTTCCACCATCTCCTATCGATGCAGGCCGGGTC  900
  I  C  G  G  Y  N  T  P  T  H  N  P  P  V  P  Q  H  D  H  L  T  S  F  H  H  L  L  S  M  Q  A  G  S

ITATCAGAACGGGGCCTGGTCCGCGGCCGCGGGCAAGGTGGGCATGGAGGAGAACTGCATGCTGCTCGGCCACTCCTGCATCGTGGCGCCGACCGGGGAA  1000
 Y  Q  N  G  A  W  S  A  A  A  G  K  V  G  M  E  E  N  C  M  L  L  G  H  S  C  I  V  A  P  T  G  E

ATCGTCGCTCTCACTACGACGCTGGAAGACGAGGTGATCACCGCCGCCGTCGATCTCGATCGCTGCCGGGAACTGCGTGAACACATCTTCAACTTCAAGC  1100
I  V  A  L  T  T  T  L  E  D  E  V  I  T  A  A  V  D  L  D  R  C  R  E  L  R  E  H  I  F  N  F  K  Q

AGCATCGTCAGCCCCAGCACTATGGTCTGATCGCGGAACTCTGAGGTTGCCGAAAAGCATGTGTGTCGTTGTTCTCGGCGCCTGGGTCACATCCAGGCGC  1200
  H  R  Q  P  Q  H  Y  G  L  I  A  E  L

GCCAGGGTGACGCTGGTGGAATAGTACCACGACCGCTTCAGGGCGATCCGCAAGGAGATGCGGGTCGCCGGAGCGGCAAAGCCCGACATTCGTTTCGCAC  1300

CGACGGCCGTCGTGAACTCGACAGTCCGCGAGAAGGGCGTATTGCGCGGCCTGGACCTGTACGTGGAACTGTAGCCCATATATAGATTTCCAAAGAGTTT  1400

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GCACTTGAGAATGCGATCGCCATCGTCGATTACATCAACGGTACACCGCCCCATATCGCGTCCCTGGCGGAGCTTTCAACGACGCTCGGGATATCCAAGA  1700

GCCACTGTCACTCCATCCTCAAGACGCTGACGCATTTCGGCTGGCTGAAATTCGACAATCGCTCAAAGAGCTACGAGCTGAATTC
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Fig. 4. Nucleotide Sequence of the DCase Gene from *Agrobacterium* sp. Strain KNK712 and Its Deduced Amino Acid Sequence.

The underline shows the open reading frame of the DCase gene. The nucleotide sequence data appears in the DDBJ, EMBL and Gene Bank databases under the accession number AB007368.

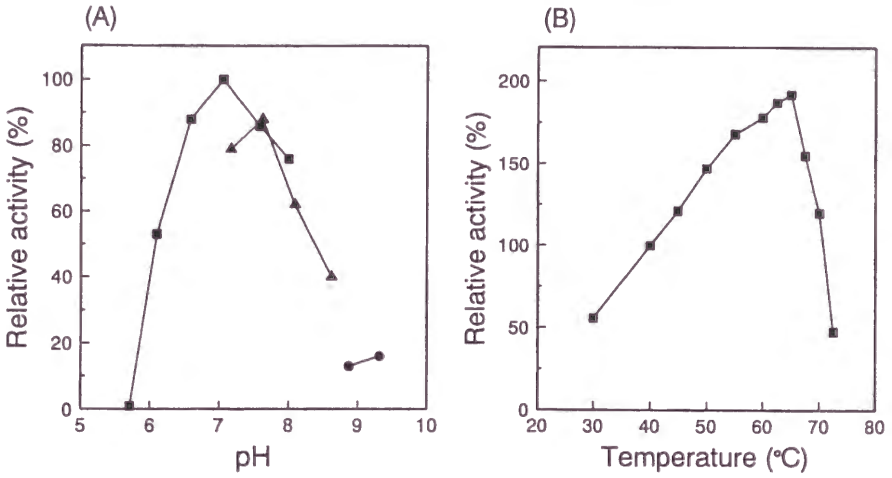

Fig. 5. Effects of pH (A) and Temperature (B) on the Activity of DCase.

A cell extract of *E. coli* JM109(pAD108) was used. (A) The enzyme activity was assayed under the standard assay conditions except that the following buffers were used at 100 mM : potassium phosphate, pH 5.7-8.0 (■); Tris-HCl, pH 7.2-8.6 (▲); and carbonate, pH 8.9-9.3 (●). (B) Assays were at various temperatures under the standard assay conditions. The relative activity is the percentage of the activity at 40°C, and pH 7.0.

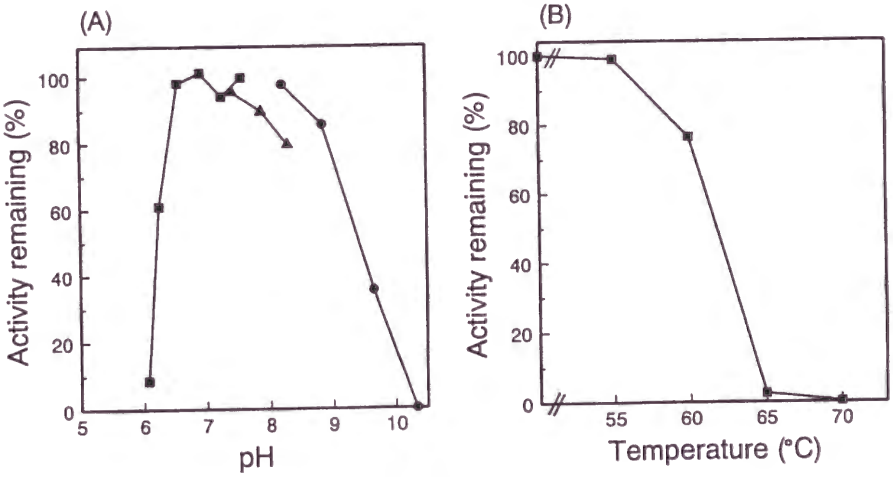

Fig. 6. Effects of pH (A) and Temperature (B) on the Stability of the DCase.

A cell extract of *E. coli* JM109(pAD108) was used. (A) 4.5 units of DCase was incubated at 40°C for 5 h in 100 mM buffers: potassium phosphate, pH 6.1-7.6 (■); Tris-HCl, pH 7.4-8.3 (▲); carbonate, pH 8.2-10.3 (●). The activity remaining was assayed under the standard assay conditions. (B) The activity remaining was assayed under the standard assay conditions after the enzyme (1.3 units) had been incubated at different temperatures for 10 min with 100 mM potassium phosphate, pH 7.0. The activity remaining is the percentage of the activity before the incubation.

The substrate specificity of the enzyme is shown in Table 3. The enzyme had broad substrate specificity for *N*-carbamylamino acids, but did not hydrolyze *N*-carbamyl-β-alanine, *N*-carbamyl-sarcosine, *N*-formyl-D-phenylalanine, or the *N*-acetylamino acids tested. In addition, it did not hydrolyze *N*-carbamyl-L-*p*-hydroxyphenylglycine, *N*-carbamyl-L-alanine, *N*-carbamyl-L-valine, or *N*-carbamyl-L-phenylalanine, although the D-isomers or racemates of these *N*-carbamylamino acids acted as substrates. The enzyme had strict D-form stereoselectivity toward these *N*-carbamylamino acids.

Table 2. Effects of SH Reagents and Metals on DCase activity.

Reagent	Concentration (mM)	Activity remaining (%)
None		100
5-5'-Dithiobis (2-nitrobenzoic acid)	5	0
Iodoacetic acid	5	0
N-Ethylmaleimide	5	0
p-Chloromercuribenzoic acid	0.1	8
$Na_2S_4O_6$	1	0
$CuSO_4$	1	0
$HgCl_2$	1	0
$AgNO_3$	1	0
$PbCl_2$	1	94
$FeSO_4$	1	93
$FeCl_3$	1	99

The enzyme in the form of a cell extract of *E. coli* JM109(pAD108) was incubated with a reagent in potassium phosphate buffer (pH 7.0) at 30°C for 60 min. The activity remaining was determined under the standard assay conditions.

Table 3. Substrate Specificity of the DCase.

Compound	Activity (%)	Assay method
N-Carbamyl		
-D-*p*-hydroxyphenylglycine	100	A
-D-phenylglycine	110	A
-DL-tryptophan	42	A
-DL-threonine	27	A
-DL-serine	55	A
-glycine	12	A
-D-alanine	81	B
-D-valine	38	B
-D-leucine	100	B
-D-methionine	160	B
-DL-isoleucine	72	B
-DL-phenylalanine	53	B
-DL-aspartic acid	6	B
-DL-norvaline	76	B
-DL-norleucine	105	B
-DL- α -amino-*n*-butyric acid	110	B

A compound (D, 1% w/v; DL, 2% w/v) was incubated under the standard assay conditions with the purified DCase. Method A: ammonia release was measured. Method B: D-amino acids were assayed by the D-amino acid oxidase method described in Materials and Methods.

The following compounds did not serve as substrates under the standard assay conditions except when incubation was for 150 min: *N*-carbamyl-L-*p*-hydroxyphenylglycine, *N*-carbamyl-L-alanine, *N*-carbamyl-L-valine, *N*-carbamyl-L-phenylalanine, *N*-carbamyl-sarcosine, *N*-carbamyl- β -alanine, *N*-formyl-D-phenylalanine, *N*-acetyl-DL-alanine, *N*-acetyl-DL-methionine, and glycyl-DL- alanine. The corresponding amino acids were assayed by thin-layer chromatography.

DISCUSSION

With the overexpressed DCase, the enzymatic production of D-amino acids was efficient and economical. Overexpression of the DCase from *Agrobacterium radiobacter* NRRL B11291 in *E. coli* cells has been reported previously.[18, 19] Compared with the specific activity of the cell extract reported there, the specific activity of our cell extract was about 5 times higher, probably because the host-vector system and the culture conditions for the recombinant *E. coli* used here were more suitable for the expression of DCase.

Sequences of the DCase of *Agrobacterium* sp. strain KNK712 and that of *Agrobacterium radiobacter* NRRL B11291[18] were similar, but nine residues of the deduced amino acid sequence were different in the two DCases.[35] Therefore, the two DCases were not identical. The N-terminal sequence of 20 amino acids of the DCases of *Agrobacterium* sp. strain KNK712 and four other strains, *Agrobacterium radiobacter* NRRL B11291,[18] another *Agrobacterium* sp.,[16] a *Comamonas* sp.,[15] and a *Blastobacter* sp.,[14] were 100%, 100%, 40%, and 65% similarity, respectively. These results indicated that the DCases of *Agrobacterium* sp. strain KNK712, *Agrobacterium radiobacter* NRRL B11291,[18] and the other reported *Agrobacterium* sp.[16] are similar.

The molecular weight of a subunit was 34,285, which was almost the same as the molecular weights of the subunits of the DCases of the two other *Agrobacterium* strains already reported.[16, 18] The subunits were slightly smaller than the subunits of the DCases of *Comamonas*[15] and *Blastobacter*,[14] with molecular weights of 40,000.

The DCase obtained was more stable than the DCases of *Comamonas* and *Blastobacter* reported by Ogawa *et al.*[15, 16] Thermostability is advantageous for the production of D-amino acids.

The optimum pH and pH giving stability were both about pH 7, the same as for *Agrobacterium radiobacter*,[10, 19] the other *Agrobacterium* sp. studied,[16, 17] and *Pseudomonas* sp. strain KNK003A.[35] They were different from the *Comamonas* sp.[15] and *Blastobacter* sp.,[14] which had alkaline optimum pHs.

The DCase obtained was inhibited by SH reagents, as were the DCases reported by Ogawa *et al.*,[14, 15] Olivieri *et al.*,[10] Buston *et al.*,[19] and Louwrier *et al.*,[16] and was stabilized by DTT, so that the DCase had an SH group in its active site. This DCase was inhibited by NH_3, one of its products, as Olivieri *et al.*,[10] Ogawa *et al.*,[14, 15] and Louwrier *et al.*[16] have reported for the DCase they studied.

Agrobacterium sp. strain KNK712 had not only DCase but also D-hydantoinase. D-Specific hydantoinase is identical with dihydropyrimidinase (also EC 3.5.2.2), which functions in the pyrimidine degradation pathway, and the D-hydantoinase of strain KNK712 was indeed dihydropyrimidinase (unpublished results). The genes for the two enzymes were cloned simultaneously because they are exist close together on the chromosomal DNA. However, to judge from the substrate specificity, the DCase probably did not to cooperate with the hydantoinase in pyrimidine degradation. The DCase was specific for *N*-carbamyl-D-amino acids, unlike β-ureidopropionase (EC 3.5.1.6), which hydrolyzes *N*-carbamyl-β-alanine. The DCase did not hydrolyze *N*-carbamyl-sarcosine, suggesting that it was not *N*-carbamyl-sarcosine amidohydrolase. Furthermore, *N*-formyl-amino acid, *N*-acetyl-amino acid, and Gly-Ala were not hydrolyzed by the enzyme. The DCase purified from *Agrobacterium* sp. strain KNK712 had no ureidosuccinase, ornithine carbamyltransferase, or aspartate carbamyltransferase activity (unpublished results). The same results were reported earlier for *Comamonas* sp.[15] and *Blastobacter* sp.[14] by Ogawa *et al.* The physiologic function of DCase and its relationship with hydantoinase remain uncertain.

The DCase had a broad substrate specificity for *N*-carbamyl-D-amino acids, and could efficiently hydrolyze several *N*-carbamyl-D-amino acids, but it did not hydrolyze *N*-carbamyl-L-amino acids at all. The strict D-form stereoselectivity is a useful characteristic for the production of D-amino acids.

I achieved high production of the DCase, obtained from a natural source and with properties suitable for industrial use. The DCase might be best used immobilized in a bioreactor.

SUMMARY

Agrobacterium sp. strain KNK712, which produced DCase was isolated from soil. The bacterium had D-specific hydantoinase activity also. Both enzymes are suitable for use in the production of D-amino acids. The DCase gene from *Agrobacterium* sp. strain KNK712 was cloned into *E. coli*. The cloned DNA fragment contained one open reading frame, predicted to encode a peptide of 304 amino acids, with a calculated molecular weight of 34,285. The DCase gene was overexpressed under the control of the *lac* promoter, and DCase accounted for 50% of the soluble protein in the cells. The enzyme was purified and some properties were investigated. Both the optimum pH and the pH that gave greatest stability were about pH 7.0. The optimum temperature was 65℃, and the enzyme was stable at 55℃. The enzyme had strict specificity toward *N*-carbamyl-D-amino acids, and was inhibited by thiol reagents, Cu^{2+}, Hg^{2+}, Ag^{+}, and ammonia.

CHAPTER II

Immobilization of *N*-Carbamyl-D-Amino Acid Amidohydrolase

In the previous chapter, I described a study involving the screening for DCase-producing microorganisms, the cloning of the gene, and some properties of the enzyme. The DCase of *Agrobacterium* sp. strain KNK712 catalyzed the hydrolysis reaction efficiently, and was highly produced by recombinant *E. coli* cells. Thermotolerant *Pseudomonas* species that produce thermotolerant DCase have also been reported by Ikenaka *et al.*[35] Other groups also have reported studies on this enzyme.[6, 10-19] If the DCase is immobilized, the production process for D-amino acids will be improved, as in the cases of immobilized aminoacylase,[21] penicillin acylase,[22] glucose isomerase[23] and so on, which have been used commercially for production processes in the food and pharmaceutical industries. But only Olivieri *et al.*[11] reported the immobilization of DCase in immobilized whole cells of *Agrobacterium*, which contained not only DCase but also hydantoinase. The immobilization of cell-free DCase, with which the expression of high activity without a membrane barrier is expected, has not been reported yet. Furthermore, hydantoinase and DCase have different optimum and stable pHs. To express high activity and to use them stably during long term repeated reactions, it seems better that the two enzymes are immobilized separately, and used for each step of D-amino acid production under the respective optimal conditions. So I decided to study the immobilization of DCase in a cell-free form for practical use for the decarbamylation step, which follows the reaction with immobilized hydantoinase, which has already been used in practice.[20] It can be expected that use of a bioreactor for the production will make the process simple, efficient, and highly productive, and that the economical merits will increase. In this chapter, I describe the immobilization of DCase produced by *E. coli* cells using a cloned gene from *Agrobacterium* sp. strain KNK712, its stabilization, and its use for repeated batch reactions. I also describe comparison of the stability of immobilized DCase from *Agrobacterium* sp. strain KNK712 with that of those from thermotolerant bacteria,[35] which

are expected to produce stable enzymes.

MATERIALS AND METHODS

Microorganism. *Agrobacterium* sp. strain KNK712 and *E. coli* JM109 cells carrying the recombinant plasmid pAD108, containing the DCase gene from *Agrobacterium* sp. strain KNK712, were described in Chapter I . *Pseudomonas* sp. strain KNK003A and *Pseudomonas* sp. strain KNK505 were reported by Ikenaka *et al.*[35]

Media and culture conditions. Recombinant *E. coli* JM109 cells were aerobically cultured in 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) with 100 $\mu g \cdot ml^{-1}$ of ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside in a 2-liter shaking flask at 37℃ for 16 h. *Agrobacterium* sp. strain KNK712 was cultured as described in Chapter I . *Pseudomonas* sp. strain KNK003A and *Pseudomonas* sp. strain KNK505 were cultured under the conditions reported by Ikenaka *et al.*[35]

Chemicals. *N*-Carbamyl-D-*p*-hydroxyphenylglycine was prepared from D-*p*-hydroxyphenylglycine.[28] Duolite (Rohm and Haas Ltd.), Amberlite (Rohm and Haas Ltd.), Diaion (Mitsubishi Chemicals), and Chitopearl (Fuji Spinning Co., Ltd.) were used as the supports for immobilization.

Other chemicals used in this work were the best available commercial products.

Screening of the resins for immobilization. *E. coli* JM109 (pAD108) cells were harvested from 500 ml of culture broth by centrifugation (3000 x g, 5 min, 4℃), suspended in 25 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM DTT, and then disrupted by sonication. The cell debris was removed by centrifugation (18000 x g, 10 min, 4℃). The supernatant was heated at 55℃ for 20 min. After centrifugation, the supernatant was obtained as the enzyme solution. Each resin was washed with water, and then

equilibrated with 0.01 M and 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM DTT at 4℃ for 18 h. After filtration, 250 mg of each equilibrated resin, 0.56 ml of an enzyme solution containing 10 mg of protein, and 2 ml of equilibration buffer were mixed, and then shaken at 4℃ for 18 h. After adsorption, each resin was washed with the equilibration buffer, sealed under nitrogen, and then incubated at 40℃. Before and after incubation, the activity of each resin was assayed.

Enzyme immobilization. The enzyme extract from *E. coli* JM109 (pAD108) was prepared in the way described above except for 10-fold concentration from the culture broth, using 5 mM DTT, and no heat treatment. Duolite A-568 was washed with 1 M NaCl, water and then 0.1 M potassium phosphate buffer (pH 7.0), and equilibrated with the same buffer for 18 h at room temperature, and then 213.2 g of the wet equilibrated resin was prepared from 100 g of dry resin by filtration. To the enzyme extract, 7.7 g of equilibrated resin (40 mg-protein/g-wet-resin) and DTT (final concentration, 5 mM) were added. This mixture was stirred at 4℃ for 20 h to adsorb the enzyme under nitrogen sealing. After adsorption, the resin was washed three times with a 5-fold volume of 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM DTT, crosslinked with a 5-fold volume of 0.1% or 0.2% glutaraldehyde/0.1 M potassium phosphate buffer (pH 7.0) at 4℃ for 10 min, washed three times with a 5-fold volume of 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM DTT at 4℃, and then collected by filtration.

The immobilization of DCase of *Agrobacterium* sp. strain KNK712, *Pseudomonas* sp. strain KNK003A, and *Pseudomonas* sp. strain KNK505 was as follows. Enzyme solutions of the two *Pseudomonas* were prepared as for *E.coli*JM109 (pAD108) except heating at 65 ℃ for strain KNK003A or 70 ℃ for strain KNK505 for 20 min. after sonication. For adsorption, the ratio of the weight of the wet resin and that of protein in the enzyme solution were 1 to 0.025. The resins were crosslinked with 0.08% glutaraldehyde at room temperature for 30 min. DTT was not used for preparation of enzyme solution and immobilization. Other conditions were the same as those for *E. coli* JM109 (pAD108).

Enzyme assay. Soluble DCase was assayed by the method described in Chapter Ⅰ. Immobilized DCase was assayed by measurement of D-*p*-hydroxyphenylglycine produced from *N*-carbamyl-D-*p*-hydroxyphenylglycine. The reaction was started by the addition of 100 mg of wet immobilized enzyme to the assay mixture comprising 190 μ mol of *N*-carbamyl-D-*p*-hydroxyphenylglycine and 400 μ mol of potassium phosphate buffer, pH 7.0, in a total volume of 4 ml. After 10 min of incubation with stirring at 40℃, the reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid. The D-*p*-hydroxyphenylglycine was analyzed by HPLC. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of D-*p*-hydroxyphenylglycine at the rate of 1 μ mol·min^{-1} under the assay conditions mentioned above.

Effects of reductants. After DCase of *E. coli* JM109 (pAD108) immobilized on Duolite A-568 (37 units, 0.5 g) had been put onto a column (11 x 150 mm), 2% of *N*-carbamyl-D-*p*-hydroxyphenylglycine solution containing 1 mM reductant (pH 6.9): DTT, L-cysteine, cysteamine, or sodium hydrosulfite, or without reductant, was put on the column for continuous hydrolysis, at 1 ml·min^{-1}, at 40℃ under nitrogen sealing for 4 days. The conversion of the substrate was analyzed with HPLC.

Repeated batch reactions with the immobilized DCase. Using the immobilized DCase of *E. coli* JM109 (pAD108), repeated batch reactions were done as follows. To prepare a substrate solution, 3 g of *N*-carbamyl-D-*p*-hydroxyphenylglycine was added to 100 g of deionized water bubbled with nitrogen gas at 40℃, and then the pH was adjusted to 7.0 by the addition of 3 g of 6 M NaOH with stirring. The DCase immobilized on Duolite A-568 and the prepared substrate solution were stirred at 40℃ under a stream of nitrogen gas while the pH was controlled at 7.0 with 2 M HCl. Samples were taken for activity measurement at10 and 60 min, the reaction being continued for 23.5 h in total. The reaction mixture was removed by suction, after which fresh substrate solution was introduced and allowed to react in the same manner as described above.

Repeated batch reactions with the immobilized DCase of *Agrobacterium* sp. strain KNK712, *Pseudomonas* sp. strain KNK003A, and *Pseudomonas* sp. strain KNK505 were done as described above except that 1 g of *N*-carbamyl-D-*p*-hydroxyphenylglycine was added, the temperature of the reaction was 45℃ for the immobilized enzymes of *Pseudomonas* sp. strain KNK003A and *Pseudomonas* sp. strain KNK505, the pH was controlled at 6.9 with 0.5 M HCl, and samples were taken for the calculation of the activity at 23.5 h. Considering the effect of adsorption of produced D-*p*-hydroxyphenylglycine to the immobilized enzyme on analysis, the data of the first batch was neglected, and the remaining activity was expressed as a percentage of the amount of the product in the second batch.

Analytical methods. All analytical methods were described in Chapter Ⅰ.

RESULTS

Screening of resins for immobilization

Using DCase of *E. coli* JM109 (pAD108), resins for immobilization were screened. Porous polymers such as Duolite A-568, Diaion HPA 25, Chitopearl 2503, and Chitopearl 3003 had higher adsorbed DCase activity in 0.01 M potassium phosphate buffer among 23 resins (Table 4). On the other hand, in 0.1 M potassium phosphate buffer, only Duolite A-568 and Chitopearl 3003 had high adsorbed DCase activity. The other two resins, which had high adsorbed DCase activity in 0.01 M potassium phosphate buffer, had no adsorbed enzyme activity, suggesting that adsorbing ability decreases in the high ionic strength buffer. DCase adsorbed on Duolite A-7 had lower activity than that on Duolite A-568, but it also showed some activity in both 0.01 M and 0.1 M potassium phosphate buffer. DCase adsorbed on Duolite A-568, Duolite A-7, and Chitopearl 3003 gradually lost their activity on incubation at 40℃ for 4 days, suggesting that their stability is not high enough.

Considering activity and stability of the DCase, and commercial reasons, we selected Duolite A-568 as the best resin for immobilization of the DCase.

Table 4. Activity and Stability of the Immobilized DCase.

Resin	Support	Functional group	Remaining activity (units/g-resin)					
			0.01 M KPB			0.1 M KPB		
			0 day	1 day	4 days	0 day	1 day	4 day
Duolite								
A-7	P-F	secondary amine	55	38	31	32	27	14
A-561	P-F	tertiary amine	50	37	36	2		
A-568	P-F	tertiary amine	96	62	51	84	54	44
A-161	S-DVB	quaternary ammonium	41	38	37	4		
A-368	S-DVB	tertiary amine	9	9	7	6		
A-378	S-DVB	tertiary amine	76	63	52	0		
S-587	P-F	amine	26	16		31	24	18
XAD-761	P-F	hydroxy	4					
S-861	S-DVB	none	0					
Amberlite								
IRA-35	A-DVB	tertiary amine	13	11		0		
IRA-904	S-DVB	quaternary ammonium	77	70		0		
IRA-93ZU	S-DVB	tertiary amine	59	49		5	3	
Diaion								
WA 10	A-DVB	tertiary amine	0			0		
WA 21	S-DVB	primary, secondary amine	0			0		
WA 30	S-DVB	tertiary amine	32	23	15	5		
HPA 25	S-DVB	quaternary ammonium	112	109	108	0		
PA 308	S-DVB	quaternary ammonium	17	13		10	6	
SA 11A	S-DVB	quaternary ammonium	0			0		
HP 20	S-DVB	none	3			4		
Chitopearl								
BCW 2503	C	quaternary ammonium	129	123		0		
BCW 2603	C	tertiary amine	73	69		0		
BCW 3003	C	primary amine	88	43		91	66	47
BCW 3503	C	primary amine	26	3		35	7	

Abbreviations: A-DVB, acrylic-divinylbenzene; P-F, phenol-formaldehyde; S-DVB, styrene-divinylbenzene; C, chitosan; KPB, potassium phosphate buffer.

Table 5. Immobilization of the DCase on Duolite A-568.

exp.	GA treatment	Activity put on	Protein put on	Activity adsorbed	Activity		Activity yield	Protein adsorbed	Protein yield
					before GA	after GA			
	(%)	(units/g-resin)	(mg/g-resin)	(units/g)	(units/g)	(units/g)	(%)	(mg/g)	(%)
1	0.1	100	40	92	42	35	35	31	78
2	0.2	97	40	88	42	11	11	31	78

The DCase activity of the enzyme solution in experiments 1 and 2 was 17 units/ml and 16 units/ml, respectively. The protein concentration of the enzyme solution in experiments 1 and 2 was 6.8 mg/ml.

Activity adsorbed = (Activity put on)-(Remaining activity in the supernatant after adsorption)

Activity yield = (Activity after GA)/(Activity loaded) x 100

Protein adsorbed = (Protein put on)-(Remaining protein in the supernatant after adsorption)

Protein yield = (Protein adsorbed)/(Protein put on) x 100

Abbreviations: exp, experiment; GA, glutaraldehyde

Immobilization on Duolite A-568

Using a cell-free extract from *E. coli* JM109 (pAD108), DCase was immobilized on Duolite A-568 by adsorption and crosslinking (Table 5). Its activities in experiments 1 and 2 amounted to 35 units/g-wet-resin and 11 units/g-wet-resin, and the activity yields of experiments 1 and 2 were 35% and 11%, respectively. DCase activity decreased on crosslinking with glutaraldehyde, and so the activity of the highly crosslinked immobilized enzyme in experiment 2 was one-third that in experiment 1. About 10% of the activity put on and about 20% of the protein put on remained in the supernatant after adsorption, and thus were not immobilized.

pH stability

The effects of pH on the stability of the immobilized DCase produced with *E. coli* JM109 (pAD108) were investigated. The immobilized enzyme was most stable at around pH 7.0 at 40℃ for 48 h. The immobilized enzyme was more stable in Tris-HCl buffer than in potassium buffer (Fig. 7).

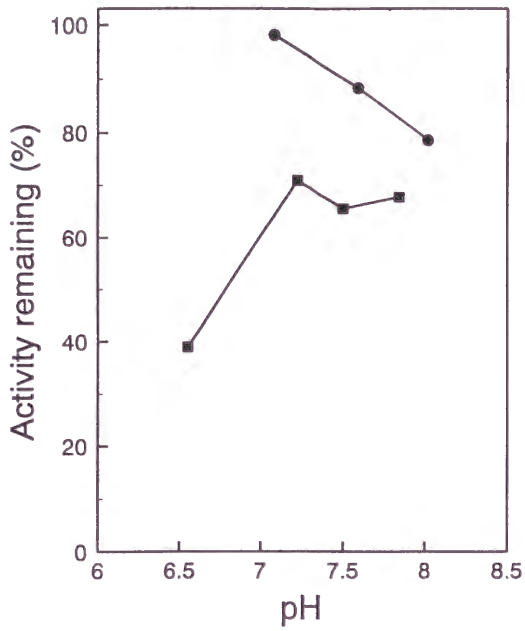

Fig. 7. Effects of pH on the Stability of the Immobilized DCase.

DCase immobilized on Duolite A-568 (0.8g) was incubated at 40℃ for 48 h in 100 mM buffers containing 10 mM dithiothreitol: potassium phosphate, pH 6.55-7.84 (■); Tris-HCl, pH 7.08-8.02 (●). After this was washed with 100 mM potassium phosphate buffer containing 10 mM dithiothreitol, pH 7.0, the remaining activity was assayed under the standard assay conditions, and was expressed as a percentage of the activity before incubation.

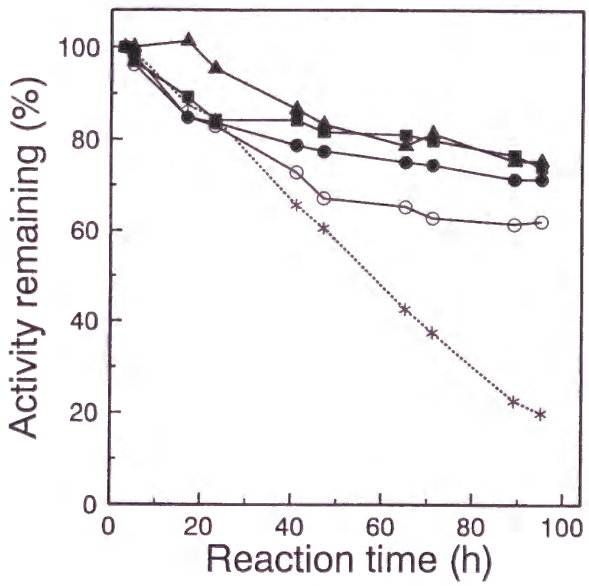

Fig. 8. Effects of Reductants on the stability of the immobilized DCase.

Reductants used were dithiothreitol (▲), cysteine (●), cysteamine (■), sodium hydrosulfite (○), or none (*).

Effects of reductants

Using immobilized DCase produced with *E. coli* JM109 (pAD108), the continuous column reaction was done with or without reductant (Fig. 8). Under conditions described in Materials and Methods, initial conversion of substrate to the product with each reductant or without it was about 10%, and then gradually decreased in conversion because of the loss of the enzyme activity. However, with the use of reductants such as DTT, L-cysteine, cysteamine and sodium hydrosulfite during the continuous reaction, the degree of enzyme inactivation in 4 days was suppressed to 30% to 45%, though it was 80% when no reductant was used.

Repeated batch reactions

Figure 9 shows the results of repeated batch reactions with immobilized DCase of *E. coli* JM109 (pAD108). The reaction stability was greatly improved when DTT was present in the reaction mixture, and a higher crosslinking degree also stabilized the immobilized enzyme. The remaining activity of the immobilized enzyme crosslinked with 0.1% and 0.2% glutaraldehyde, and 0.2% with DTT in the reaction mixture was 22%, 38%, and 84% after 4 times repeated reactions, and 12%, 18%, and 63% after 14 times repeated reactions, respectively. When the activity had decreased to about 40% of the initial level, the substrate began not to be converted to D-p-hydroxyphenylglycine completely in 23.5 h.

DCase from *Pseudomonas* sp. strain KNK003A and *Pseudomonas* sp. strain KNK505, which are thermotolerant bacteria, and that from *Agrobacterium* sp. strain KNK712 were immobilized on Duolite A-568, and the stability during repeated batch reactions was examined (Fig. 10). The stabilities of the immobilized enzymes of the two thermotolerant bacteria were superior to that of *Agrobacterium* sp. strain KNK712, though the former two were used at higher temperatures. However, their activity was lower than that of *Agrobacterium* sp. strain KNK712. Compared to the activity of immobilized DCase of *E. coli* JM109 (pAD108), even that of *Agrobacterium* sp. strain KNK712 was twenty times lower. Low activity of these immobilized enzymes was probably caused by the low specific activity

of the enzyme solutions used for immobilization. Activity of the three enzymes shown in Fig. 10 were too low to convert substrate to D-*p*-hydroxyphenylglycine completely during the reaction.

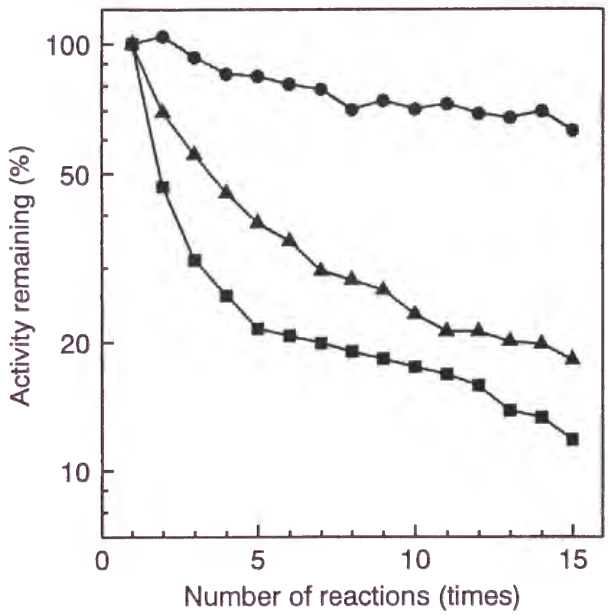

Fig. 9. Stability of the Immobilized DCase of *E. coli* JM109 (pAD108) on Repeated Batch Reactions.

Repeated batch reactions were done as described under Materials and Methods using the immobilized DCase crosslinked with 0.1% (■) or with 0.2% glutaraldehyde (▲), or 0.2% glutaraldehyde with dithiothreitol in the reaction mixture (●).

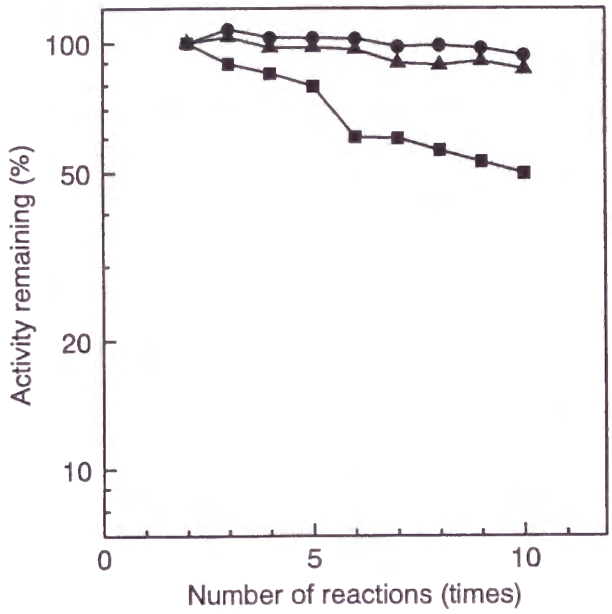

Fig. 10. Stability of the Immobilized DCases of *Agrobacterium* sp. Strain KNK712 and Thermotolerant Bacteria on Repeated Batch Reactions.

Repeated batch reactions were done as described under Materials and Methods using the immobilized DCase of *Agrobacterium* sp. strain KNK712 (■), *Pseudomonas* sp. strain KNK003A (▲), or *Pseudomonas* sp. strain KNK505 (●).

DISCUSSION

To use the immobilized enzyme practically, the activity of it must be high and stable during long term reaction. To satisfy these points, I examined methods of immobilization. I screened suitable supports for immobilization, of which the activities were high and stable under both low and high ionic strength conditions. The majority of the immobilized enzymes showed some activity in the low ionic strength buffer, but low activity in the high ionic strength buffer, suggesting that the protein was held on to the resin through ionic bonds. But among these resins, Duolite A-568, Duolite A-7, and Chitopearl 3003 showed high adsorbed

DCase activity even under high ionic strength conditions, suggesting that these resins hold the protein through not only ionic bonds but also through hydrophobic bonds between the protein and the resin. In an actual reaction, the immobilized enzyme is under high ionic strength conditions, so a resin on which the enzyme is immobilized stably under high ionic strength conditions can be used more stably in the reaction. From the activity and stability of the enzyme, and commercial reasons, I selected Duolite A-568 as the most suitable for immobilization of the DCase.

The activity yield on the immobilization of DCase on Duolite A-568 was 11-35%. The first reason for the low yield is that DCase is sensitive to glutaraldehyde. As DCase might be crosslinked strongly, it lost much more activity, suggesting that the reaction between glutaraldehyde and the amino groups around the active site causes the activity loss. The second reason is that Duolite A-568 is a macroporous resin (average pore size, about 200 Å). It is supposed that the limitation of diffusion of a substrate through the pores causes the decrease in activity of the immobilized enzyme when the activity is measured at the same substrate concentration as for the soluble enzyme. In practice, the apparent *K*m of the immobilized enzyme was 30 mM (data not shown), which was larger than that of the soluble enzyme, 0.89 mM (Chapter Ⅰ). Thirdly, the yield of the adsorbed protein was 78%. In late period of adsorption, it is proposed that the rate of adsorption decreases because of the decrease in protein concentration, and the adsorbing capacity of the resin was filled.

The stability of the immobilized enzyme was studied. The most stable pH was around 7, which was the same as that for the soluble DCase (Chapter Ⅰ). Reductants such as DTT, L-cysteine, cysteamine, and sodium hydrosulfite stabilized the immobilized enzyme during the continuous reactions. As I described in Chapter Ⅰ, soluble DCase also stabilized by DTT, and inhibited by SH reagents. DCase seemed to be an SH enzyme, so it can be supposed that the enzyme is denaturated through oxidation of the SH groups at the active site or other SH groups.

The stability on repeated batch reactions was also remarkably improved by using DTT as a reductant. Among the many reasons for the inactivation, the most important point as to

repeated reaction stability was prevention of oxidation of the enzyme in this case. Glutaraldehyde also improved the stability, suggesting that the enzyme released from the resin decreased, and the enzyme steric conformation was stabilized by enzyme-resin and enzyme-enzyme crosslinking. But little loss of activity occurred in spite of the reduction with DTT and high crosslinking. Considering these results, reduction and crosslinking were not enough, and denaturation due to heat and acid for the control of pH also might occur.

By using recombinant *E. coli* which could produce DCase copiously, an immobilized enzyme with high activity and stability was prepared in this study, and probably it can be actually used. Further, I found that immobilized DCases of thermotolerant bacteria were quite stable during reaction, though their activity was low. Ikenaka *et al.*[35] reported that these enzymes showed high thermostability. Though there are many reasons for inactivation in the repeated reactions, it can be proposed that the use of a thermotolerant enzyme is an effective means of improving stability. Studies along such lines to create a more highly active and stable immobilized DCase will be done.

SUMMARY

N-Carbamyl-D-amino acid amidohydrolase (DCase), produced by recombinant *E. coli* cells using a cloned gene from *Agrobacterium* sp. strain KNK712, has been immobilized for use in the production of D-amino acids. The porous polymers, Duolite A-568 and Chitopearl 3003, were much better than other resins for the activity and stability of the adsorbed enzyme. The activity of DCase expressed on Duolite A-568 and Chitopearl 3003 amounted to 96 units·g^{-1}-wet-resin and 91 units·g^{-1}-wet-resin, respectively. DCase immobilized on Duolite A-568 was found to be most stable at about pH 7, and it was further stabilized by reductants such as DTT, L-cysteine, cysteamine, and sodium hydrosulfite. The stability during the repeated batch reactions was greatly improved when DTT was in the reaction mixture, and the higher crosslinking degree with glutaraldehyde also stabilized the immobilized enzyme.

After 14 times repeated reactions, the remaining activity of the immobilized enzyme cross-linked with 0.1% and 0.2% of glutaraldehyde, and 0.2% of glutaraldehyde with DTT in the reaction mixture was 12%, 18%, and 63%,respectively. DCase produced by *Pseudomonas* sp. strain KNK003A and *Pseudomonas* sp. strain KNK505, which are thermotolerant soil bacteria, and that by *Agrobacterium* sp. strain KNK712 were also immobilized on Duolite A-568. The stability of the enzymes of thermotolerant bacteria during reactions was superior to that of *Agrobacterium* sp. strain KNK712, though the activity was lower than that of strain KNK712.

CHAPTER Ⅲ

Immobilization of Thermotolerant *N*-Carbamyl-D-Amino Acid Amidohydrolase

An immobilized enzyme is beneficial not only because it can be reused for the reaction, but also because it facilitates the operation and product isolation, and improves the yields of the products. Therefore, such enzymes have been used widely for industrial purposes.[21-23]

In order to improve the production of D-amino acids, I studied the application of a bioreactor to enzymatic reactions. In Chapters Ⅰ and Ⅱ, I described the screening of a DCase, cloning and overexpression of its gene from *Agrobacterium* sp. KNK712, and its immobilization. This overexpressed DCase seemed to exhibit higher activity than other previously reported ones,[6,10-19] but its stability was not enough when the immobilized enzyme was used in repeated reactions. To resolve this problem, the DCase has been improved by means of gene mutation, and some thermotolerant DCases have been obtained, in which an amino acid was substituted.[36,37,40]

In this chapter, I describe the effect of the thermostability of the improved DCase on the stability of the immobilized enzyme in repeated reactions and the characteristics of the immobilized enzyme.

MATERIALS AND METHODS

Microorganisms. *E. coli* JM109 carrying recombinant plasmid pAD108 was described in Chapter Ⅰ. *E. coli* JM109 carrying recombinant plasmid pAD402, pAD404, pAD406 or pAD416 was reported by Ikenaka *et al.*[36,37] Each plasmid has a DCase gene which codes a native or mutant DCase of *Agrobacterium* sp. KNK712. The numbers of the plasmids are those name of the DCases.

Media and culture conditions. Recombinant *E. coli* JM109 was aerobically cultured in 2YT medium (1.6% of tryptone, 1% of yeast extract, and 0.5% of NaCl, pH 7.0) containing 100 μ g/ml of ampicillin in a 2 liter Sakaguchi flask at 37℃, for 16 h.

Chemicals. *N*-Carbamyl-D-*p*-hydroxyphenylglycine was prepared from the corresponding amino acid .[28] Duolite A-568 (Rohm and Haas Ltd.) was employed as a support for immobilization.

Other chemicals used in this work were the best available commercial products.

Preparation and immobilization of DCases. Native (108) and four kinds of mutant DCases (402, 404, 406, and 416) were used for immobilization (See Table 6 for the positions of the mutations and the amino acid substitutions in each mutated enzyme). Cells of *E. coli* JM109(pAD108), JM109(pAD402), JM109(pAD404), JM109(pAD406), and JM109(pAD416) were harvested from 500 ml of cultured broth by centrifugation (3000 x g, 10 min, 4℃), suspended in 50 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM DTT, and then disrupted by sonication. The cell debris was removed by centrifugation (18000 x g, 10 min, 4℃), and then the supernatant was obtained as the enzyme solution. Duolite A-568 was washed with 1 M NaCl, water, and then 0.1 M potassium phosphate buffer, pH 7.0, and equilibrated with the same buffer for 18 h at room temperature, and then the wet equilibrated resin was prepared by filtration. The wet resin and DTT (final

concentration, 5 mM) was added to the enzyme solution, followed by stirring at 4℃ for 20 h under nitrogen sealing. For adsorption, the weight of the wet resin and that of protein in the enzyme solution were in the ratio of 1 to 0.04. After adsorption, the resin was washed three times with a 5-fold volume of 0.1 M potassium phosphate buffer, pH7.0, containing 10 mM DTT, crosslinked with a 5-fold volume of 0.2% of glutaraldehyde in 0.1 M potassium phosphate buffer, pH7.0, containing 10 mM DTT at 4℃ for 10 min, washed 3 times with a 5-fold volume of 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM of DTT at 4℃, and then filtered.

Enzyme assay. Soluble DCase and immobilized DCase were assayed at 40℃ and pH 7.0 by the methods described in Chapters Ⅰ and Ⅱ.

One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of D-*p*-hydroxyphenylglycine at the rate of 1 μ mol·min^{-1} under the assay conditions.

To determine the effects of temperature and pH on the activity or stability of immobilized DCase, standard assays were performed as follows. The reaction was started by the addition of 200 mg of wet immobilized enzyme to the assay mixture comprising 4430 μ mol of *N*-carbamyl-D-*p*-hydroxyphenylglycine, 3000 μ mol of potassium phosphate buffer, pH 7.0, and 486 μ mol of DTT, in a total volume of 30 ml. After 10 min incubation with stirring at 50℃, the reaction was stopped by the addition of 1 ml of 5 M H_2SO_4. The D-*p*-hydroxyphenylglycine formed was analyzed by HPLC as described in Chapter Ⅰ.

Analytical methods. All analytical methods were described in Chapter Ⅰ.

Repeated batch reactions. As described in Chapter Ⅱ, using *N*-carbamyl-D-*p*-hydroxyphenylglycine as a substrate solution, batch reactions were carried out repeatedly in a stirred reactor at 40℃ under a stream of nitrogen gas while the pH was controlled at 7.0 with 2 M HCl. At 10 and 60 min, samples were taken for the calculation of the activity based on the product amount of 60 min minus that of 10 min, the reaction being continued for 23.5 h in

total. After the removal of reaction mixture, the reaction was repeated in the same manner.

RESULTS

Enzyme thermostability

The thermostabilities of the native and mutated DCases are shown in Table 6. The thermostabilities of the mutated DCases having an amino acid substitution were 4.7-9.6℃ higher than that of the native one, and 416 DCase, having the substitution of 236 valine to alanine, was the most stable.

Table 6. Enzyme Thermostability.

Enzyme	Amino acid substitution	Thermostability (℃)
108(native)	none	61.8
402	His 57→Tyr	67.3
404	Pro 203→Leu	68.0
406	Pro 203→Ser	66.5
416	Val 236→Ala	71.4

Solutions of JM109(pAD108), JM109(pAD402), JM109(pAD404), JM109(pAD406), and JM109(pAD416), see Materials and Methods, were incubated at temperatures from 56℃ to 74℃ at 3℃ intervals for 10 min. The remaining activity was determined under the standard assay conditions, and thermostability was expressed as the temperature at which 50% of the activity was lost.

Immobilization of DCases on Duolite A-568

The native and mutated DCases were immobilized on DuoliteA-568 by adsorption and crosslinking with glutaraldehyde (Table 7). The immobilized 402 and 416 DCases exhibited higher activity than that of the native one, and all the mutant DCases were superior in the activity yield to the native one. But the activity yields ranged from 9.6% to 16.4%, which was not so high.

Table 7. Immobilization of DCases

Enzyme	Specific activity			Activity yield
	Loaded enzyme	Immobilized enzyme		
		before GA	after GA	
	(units/mg)	(units/g)	(units/g)	(%)
108(native)	2.43	41.8	10.8	9.6
402	2.31	37.8	15.1	16.4
404	2.02	36.8	11.5	12.8
406	2.34	36.9	11.1	11.9
416	2.51	44.5	14.6	14.6

Abbreviation; GA, glutaraldehyde.

Repeated batch reactions

The stability on repeated batch reactions in a stirred reactor was examined. When dithiothreitol was not present in the reaction mixture, all the mutant DCases were more stable than the native one. Among them, the 416 and 404 DCases showed the highest stability (Fig. 11).

Compared with the results in Fig. 11, Fig. 12 shows that the stability of the immobilized enzymes was improved when DTT was present in the reaction mixture.

Based on the results of repeated reactions with DTT (Fig. 12), the half-lives of the activity were calculated from the regression coefficients. The half-lives of the activity on reaction for 1 to 8 times were 15 time for 108 DCase, 42 times for 404 DCase and 37 times for 416 DCase, and those for 9 to 17 times were 58 times for 108 DCase and 104 times for 404 DCase. From the results shown in Fig. 12 and the calculated half-lives, the mutant 404 and 416DCases showed almost the same stability, which was superior to that of the native 108 DCase. And the activity loss was greater at early stages than late ones of the reaction, this phenomenon being observed also in case of Fig. 11.

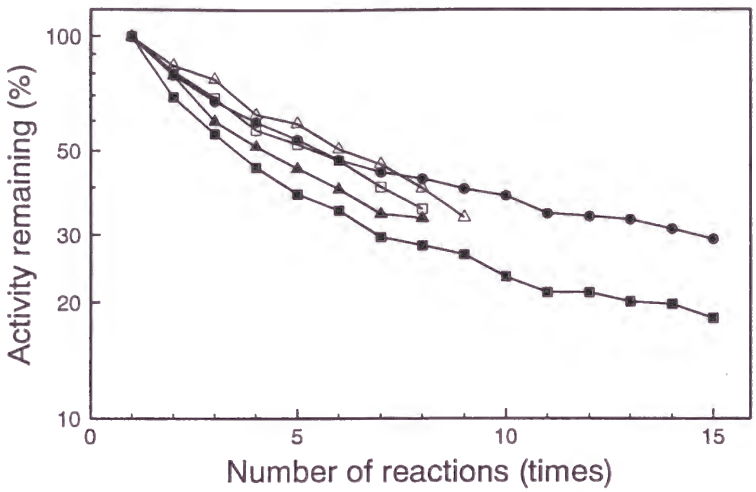

Fig. 11. Stability of Immobilized DCases on Repeated Batch Reactions without a Reductant.

Repeated batch reactions were carried out without a reductant as described under Materials and Methods using immobilized 108 (■), 402 (▲), 404 (●), 406 (□), and 416 (△) DCase. The remaining activity was expressed as a percentage of the activity in the first reaction.

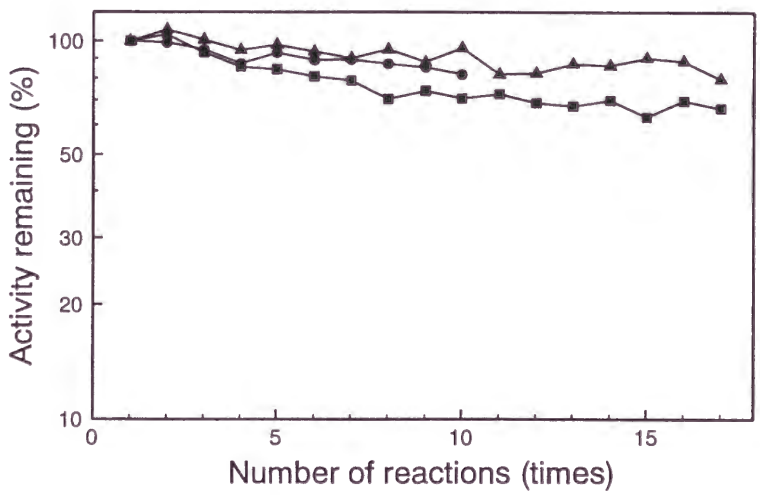

Fig. 12. Stability of Immobilized DCases on Repeated Batch Reactions with a Reductant.

Repeated batch reactions were carried out with 5 mM dithiothreitol as described under Materials and Methods using immobilized 108 (■), 404 (▲), and 416 (●) DCase. The remaining activity was expressed as a percentage of the activity in the first reaction.

Effects of temperature and pH on the activity and stability of DCase

According to an increase in the temperature, the activity of immobilized 416 DCase increased, the activity at 50℃ being 1.5 times higher than that at 40℃, as shown in Fig. 13(A). The slope of the activity of soluble 416 DCase was the same as that of immobilized 416 DCase in the range of 30℃ to 60℃, when assayed for 10 min under the standard assay conditions (data not shown).

The optimum pH was found to be about 6.5 (Fig. 13(B)), although that of soluble 416 DCase was about 7 (data not shown).

After 16 h incubation, the activities of soluble 416 DCase and immobilized 416 DCase were stable below 40℃, and remained at 25.8% and 40.2% at 60℃, respectively (Fig. 14). The immobilized 416 DCase was stable compared with the soluble enzyme.

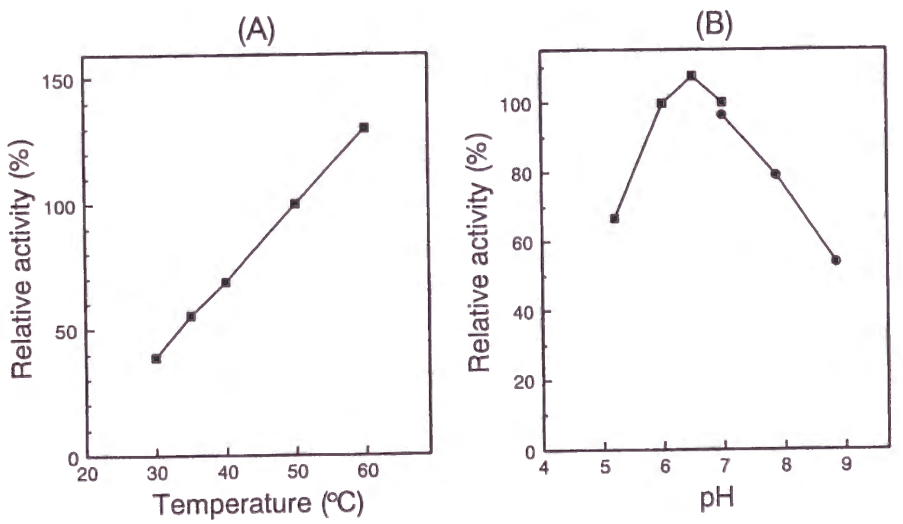

Fig. 13. Effects of Temperature and pH on the Activity of an Immobilized DCase.

Immobilized DCase from JM109(pAD416) was used. (A) The immobilized DCase was assayed at various temperatures under the standard assay conditions. (B) The immobilized DCase was assayed under the standard assay conditions except that the following buffers were used: 100 mM potassium phosphate, pH 5.2-7.0 (■); and 100 mM potassium phosphatemonobasic-50 mM sodium borate, pH 7.0-8.9 (●). The relative activity was expressed as a percentage of the activity at 50℃ and pH 7.0.

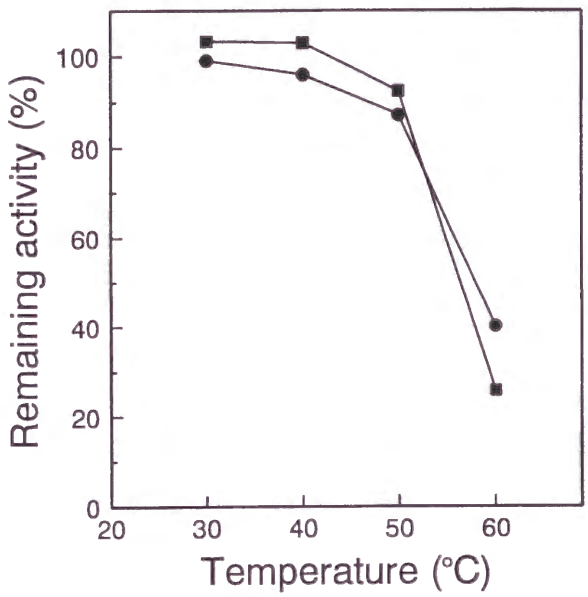

Fig. 14. Effect of Temperature on the Stability of an Immobilized DCase.

A solution of JM109(pAD416) containing 10 mM dithiothreitol (■), and 200 mg of immobilized 416 DCase in 30 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol (●) were incubated at various temperatures for 16 h. The remaining activity was determined under the standard conditions. The remaining activity was expressed as a percentage of the activity before incubation.

DISCUSSION

Thermotolerant DCases were obtained to stabilize immobilized DCases in reactions and to use them practically. They were stable when treated at high temperature, suggesting that the molecular conformations of the enzymes were stabilized by amino acid substitutions. And improvement of the thermostability caused an increase in the activity yield when they were immobilized. From the results, the thermotolerant enzyme seemed to be more resistant to denaturation due to heat, glutaraldehyde treatment or oxidation than the native one. However, the activity yield was not enough, because inactivation by glutaraldehyde was still

high, some enzyme remained in the supernatant after adsorption, and the enzyme immobilized on a support did not express complete activity because of the limitations of diffusion of the substrate into macropores.

When immobilized enzymes were used in batch reactions repeatedly, the immobilized thermotolerant DCases showed obviously improved stability compared to that of the native one. It is supposed that the inactivation was caused by heat, oxidation, and the acid used for pH control. Apparently, the conformations of the immobilized thermotolerant DCases were protected against heat effectively, and the activity seemed to be protected against other forms of denaturation.

The reductant, DTT, stabilized the activity of the immobilized enzymes effectively, suggesting that oxidation of an SH group is one of the major reasons for the inactivation. Other reductants were also able to stabilize the activity of an immobilized enzyme in reactions, as described in Chapter Ⅱ.

However, even the thermotolerant DCases gradually lost their activity when used in reactions as immobilized enzymes with a reductant and nitrogen. It was supposed that the reason was denaturation or detachment from the support due to insufficient crosslinking with glutaraldehyde and denaturation by the acid used for pH control. Inactivation occurred at early stages of the reaction and the activity gradually became stable at late stages of the reaction, suggesting that an unstable enzyme on the support was easy to denature or detach, and a stable one remained as it was.

Some loss of activity of an immobilized enzyme was observed on 16 h incubation at 50℃ or 60℃. This is because of denaturation not only by heat but also by oxidation even with a reductant. When a soluble DCase was incubated with a reductant under nitrogen sealing, it was more stable than without nitrogen (data not shown). An immobilized DCase was somewhat stable compared with the soluble enzyme, suggesting that adsorption on the support and crosslinking with glutaraldehyde stabilized the structure of the enzyme.

The activity of immobilized 416 DCase at 50℃ was 1.5 times higher than that at 40℃. An immobilized thermotolerant DCase can be used stably at a higher temperature, at which

the enzyme expresses higher activity, so that immobilized enzyme can be used more efficiently. In the range of 30℃ to 60℃, the slope for the activity of soluble 416 DCase was the same as that for the immobilized enzyme, suggesting that there was no difference in activation energy between the soluble and immobilized enzymes.

The optimum pH of an immobilized enzyme was lower than that of the soluble enzyme, suggesting that the pH around the enzyme in the macropores of the support was higher than the external pH, because the products of the DCase reaction remained in the macropores.

In this study, it was revealed that an immobilized thermotolerant DCase was more stable than the native one on repeated reactions. And it can be applied to the production of D-amino acids practically. After thermotolerant 416 DCase reported in this paper, we obtained 455 DCase, which showed further improved thermostability.[40] It was also immobilized, and exhibited excellent activity and stability. Further work on the production of this enzyme is necessary. And the mechanism of inactivation in repeated reactions should be clarified in detail.

SUMMARY

N-Carbamyl-D-amino acid amidohydrolase (DCase), in which amino acid residues were substituted by mutation, followed by the selection based on thermotolerance, showed improved thermostability, by 5 or 10℃, compared to the native DCase. These DCases were immobilized on a macroporous phenol formaldehyde resin, Duolite A-568, and the immobilized thermotolerant enzymes showed higher activity than the immobilized native DCase. From the results of repeated batch reactions, the half-lives of the activities of immobilized thermotolerant DCase, in which Leu was substituted for Pro 203, and immobilized native DCase were 104 and 58 times, respectively. It was revealed that the higher thermotolerance enabled the immobilized enzymes to be more stable in reactions. A reductant, DTT, also stabilized the enzymes in reactions. Compared with soluble DCase, immobilized DCase was somewhat stable, and its activity was optimum at a lower pH.

CHAPTER IV

Production of Thermotolerant *N*-Carbamyl-D-Amino Acid Amidohydrolase by Recombinant *Escherichia coli*

Several useful proteins have been produced by means of recombinant DNA technology using eukaryote or prokaryote cells. In particular, some bacteria are well known to be useful hosts, and a vector has been developed. Also, enzymes highly produced by these bacteria have been used as catalysts for the production of valuable chemicals, such as semisynthetic penicillins and cephalosporins.[20,38,39]

As described in the previous chapter, I tried to improve the production of D-amino acids, and thus I obtained a DCase, cloned its gene, and immobilized the enzyme, which improved its themostability.[36,37,40] Furthermore, I achieved high expression of the DCase using a recombinant *E. coli* species. Besides my work, a few groups have reported overexpression of the enzyme.[18,19] D-Amino acids can be efficiently produced using an immobilized DCase. However, the DCase activity in the cultured broth of the genetically engineered *E. coli* described in Chapter I was still not enough for economical preparation of the immobilized enzyme, although the *E. coli* produced a higher amount of DCase than other strains.[6,10-19] Furthermore, the components of the medium, such as the inducer, carbon source and nitrogen source, used for the culture in Chapter I are expensive. Another problem was that the plasmid for overexpression of the DCase was not stably maintained in the host cells. So I attempted to stabilize the plasmid and to produce a sufficient amount of DCase economically using a recombinant *E. coli* technique.

In this chapter, I describe the construction of an expression plasmid, and the hyperproduction of DCase in *E. coli* with cheap medium components.

MATERIALS AND METHODS

Microorganisms and DNA. *E. coli* used in this chapter were JM109, C600hfl, C600, LE392, AG1, K802, NM554, PLK-A, SCS1, DH1, HB101, TOP10, and DH5.

A recombinant plasmid, pAD455, carrying the DCase gene, which was cloned from *Agrobacterium* sp. strain KNK712, randomly mutated *in vitro*, selected as the gene for the enzyme whose thermostability was improved, was prepared by the methods of Chapter I and Ikenaka *et al.*[40] Plasmids pUC19 and pTrc99A were purchased from Takara Shuzo Co., Ltd., and Pharmacia Biotech, respectively.

Other molecular biological technique was the methods of Maniatis *et al.*[25]

Chemicals. Protein extract, which was a mixture of acid hydrolyzed corn gluten, wheat gluten and soybean extract, was purchased from Bansyu Chomiryo Co., Ltd. Bacto casamino acids, Bacto yeast extract and Bacto tryptone were purchased from Difco Laboratories. NZ-amine type A and corn steep liquor were purchased from Wako Pure Chemical Industries, Ltd. Bonito fillet extract type CR was purchased from Riken Vitamin Co., Ltd.

Other chemicals used in this chapter were the best available commercial products.

Media and culture conditions. Transformed *E. coli* cells were cultured on a plate of LB medium (1.0% of tryptone, 0.5% of yeast extract, 0.5% of NaCl, and 1.5% of agar, pH 7.0) containing 100 $\mu g \cdot ml^{-1}$ of ampicillin at 37℃ for 20 h. A monocolony of the recombinant *E. coli* was inoculated into and cultured in 10 ml of 2YT medium (1.6% of tryptone, 1.0%of yeast extract, and 0.5% of NaCl, pH 7.0) containing 100 $\mu g \cdot ml^{-1}$ of ampicillin in a test tube at 37℃ for an appropriate time for the selection of a host and the construction of a plasmid.

To examine the medium, glycerol stock of HB101(pNT4553) was inoculated into 10 ml of 2YT medium containing 100 $\mu g \cdot ml^{-1}$ of ampicillin in a test tube, cultured at 37℃ for 24 h, inoculated into an appropriate volume of altered standard medium A (1.5% of glycerol,

0.5% of protein extract, 0.4% of Na_2HPO_4, 0.4% of KH_2PO_4, 0.2% of NaCl, 0.05% of $MgSO_4 \cdot 7H_2O$, 4 ppm of $FeSO_4 \cdot 7H_2O$, 0.9 ppm of $ZnSO_4 \cdot 7H_2O$, 0.4 ppm of $CuSO_4 \cdot 5H_2O$, 0.2 ppm of $MnCl_2 \cdot 4H_2O$, 0.09 ppm of $Na_2B_4O_7 \cdot 10H_2O$, and 0.09 ppm of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, dissolved in deionized water and adjusted to pH 7.4 with 6 M NaOH) in a 500 ml shaking flask, and then cultured aerobically at 37℃ for 28 h. After culture for 13 h, the pH of the cultured broth was adjusted to pH 7.4 with NaOH or HCl every 3 h.

Plasmid stability. A monocolony of the transformed *E. coli* on LB medium containing 100 $\mu g \cdot ml^{-1}$ of ampicillin was inoculated into 10 ml of 2YT medium containing 100 $\mu g \cdot ml^{-1}$ of ampicillin in a test tube, and then aerobically cultured at 37℃ for 24 h. After that, the cultured broth was inoculated into 10 ml of 2YT medium in a test tube, and then aerobically cultured at 37℃ for 12 h. This culture without ampicillin was repeated a total of four times. For inoculation, 100 μl of the cultured broth was used. The fourth cultured broth was plated on LB medium, and 100 colonies were replicated onto plates of LB medium and LB medium containing 100 $\mu g \cdot ml^{-1}$ of ampicillin, respectively. Plasmid stability was expressed as a percentage of the ampicillin-resistant colonies, which formed on both LB medium and LB medium containing ampicillin.

Enzyme assay. Cells were harvested from 1 ml of cultured broth, suspended in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM of DTT, disrupted by sonication, and then assayed at 40 ℃, pH 7.0 by the method of Chapter Ⅰ. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of D-*p*-hydroxyphenylglycine at the rate of 1 $\mu mol \cdot min^{-1}$ under the assay conditions.

Analytical methods. Cell growth was measured as the absorbance at 550 nm using a spectrophotometer, U-2000 (Hitachi). Other analytical methods were described in Chapter Ⅰ.

RESULTS

Construction of an expression vector

An expression vector, pNT4553, which has the DCase gene, a *lac* promoter and the terminator on pUC19 was constructed (Fig. 15). By means of PCR methods with pUC19 (Fig. 15) as a template, and p-1 and p-2 (Fig. 16) as primers, a 1.3 Kbp HindⅢ - Cfr10 I fragment which contained an *Nde* I site was obtained. This fragment was substituted for a 1.3 Kbp HindⅢ - Cfr10 I fragment of pUC19. As a result, an *Nde* I site was introduced at the start codon of *lacZ* (pUCNde). To obtain pTrcNde, the *Nco* I site of pTrc99A was changed to an *Nde* I site as follows. By means of PCR using pTrc99A as a template, and p-3 and p-4 (Fig. 16) as primers, a 0.6 Kbp *Eco*R I - *Eco*RV fragment which contained an *Nde* I site was obtained. This fragment was substituted for a 0.6 Kbp *Eco*R I - *Eco*RV fragment of pTrc99A. Plasmid pUCNT was constructed by ligation with the 2.0 Kbp *Nde* I - *Ssp* I fragment from pUCNde and the 0.6 Kbp *Nde* I -*Ssp* I fragment from pTrcNde. Plasmid pNT4553 was obtained as follows. A *Nde* I -*Pst* I fragment of the thermotolerant 455 DCase gene was prepared by PCR using pAD455 as a template, and p-5 and p-6 (Fig. 16) as primers. Then, the 0.9 Kbp *Nde* I -*Pst* I fragment of the DCase gene and the 2.6 Kbp *Nde* I - *Pst* I fragment of pUCNT were ligated. The obtained plasmid, pNT4553, only contained an open reading frame for the DCase in pUCNT, as the sequences of primers p-5 and p-6 showed (Fig. 16).

A cell-free extract of *E. coli* JM109 transformed with pNT4553 showed high specific activity of the DCase (2.3 units·mg^{-1}-protein) without an inducer, it being 7-fold higher than that of *Agrobacterium* sp. strain KNK712. The examination of plasmid stability described under Materials and Methods showed that the stability of pNT4553 in *E. coli* JM109 was 100% at the fourth cultivation.

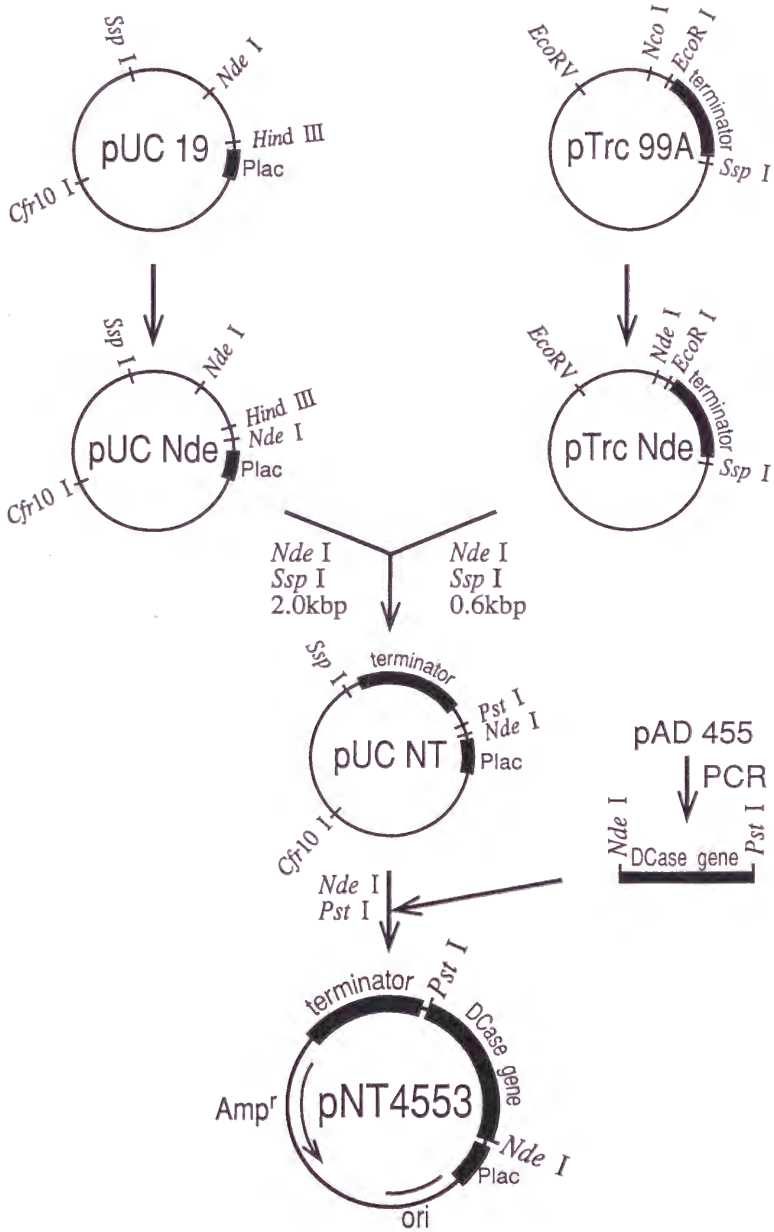

Fig. 15. Construction of Plasmid pNT4553 for Expression.

Fig. 16. Primers for PCR.

The sequences of the primers for PCR for the preparation of pUCNde (p-1, p-2), pTrcNde (p-3, p-4), and the DCase gene (p-5, p-6). The sites for the restriction enzymes and the stop codon of the DCase gene are underlined. The nucleotides for introduction of mutations are indicated by ◆.

Selection of host cells

Using pNT4553, a host for DCase production was screened (Table 8). After the introduction of pNT4553, all the *E. coli* strains except AG1, K802, PLK-A and SCS-1 expressed high specific DCase activity under the cultivation conditions in 2YT medium without an inducer. From among them, *E. coli* HB101 was selected as the best host. Its activity was 6.36 units·ml^{-1}cultured broth, which was the highest among those of the hosts examined. The specific activity of the cell-free extract without an inducer was 3.66 units·mg^{-1}-protein, which was more than 10 times higher than the specific activity of *Agrobacterium* sp. strain KNK712. On SDS-polyacrylamide gel electrophoresis,[32] the highly expressed DCase in strain HB101 (pNT4553) cells appeared in the soluble part as a main band, but no major

band of the DCase appeared in the insoluble part, suggesting that the DCase did not form inclusion bodies. The plasmid pNT4553 in HB101 was maintained stably on cultivation for 4 times under the conditions for the examination of plasmid stability given under Materials and Methods. *E. coli* NM554 also expressed high activity, and its plasmid was maintained stably. But the plasmids in eight strains of *E. coli*, some of which expressed high activity, were unstable during cultivation.

Table 8. Growth, Activity, and Plasmid Stability of the Recombinant *E. coli*.

E. coli Strain	Growth (OD550)	Total activity (units/ml)	Specific activity (units/mg)	Plasmid stability (%)
HB101	13.8	6.36	3.66	100
NM554	11.0	6.10	4.21	100
DH5	13.0	5.06	3.86	91
DH1	13.7	5.02	3.41	100
LE392	12.7	4.37	3.84	100
TOP10	10.1	4.22	3.49	95
C600hfl	8.7	3.47	5.01	97
C600	16.3	3.15	2.68	36
AG1	11.0	2.49	1.48	0
JM109	7.0	2.38	2.27	100
PLK-A	9.2	2.24	1.44	95
K802	9.6	0.72	0.53	38
SCS-1	10.2	0.07	0.05	12

Recombinant plasmid pNT4553 was used for this experiment. Total activity was expressed as units per ml of cultured broth. Specific activity was expressed as units per mg of protein. To examine plasmid stability, second cultivation broth of AG1 and SCS1 was used, and 4th broth of other strains was used.

Examination of nitrogen sources

Using glycerol as a carbon source, nitrogen and inorganic nitrogen sources for the cultivation of *E. coli* HB101(pNT4553) were examined (Table 9). When casamino acids

were used, the activity was 5.25 units·ml^{-1}, which was the best, and growth was also good. NZ-amine, tryptone and protein extract were also good for both activity and growth. When NH_4Cl or urea was used with a small amount of yeast extract, the growth was very low. Yeast extract enhanced both the activity and growth when used with protein extract.

Considering activity of the DCase and commercial reasons, I selected protein extract as the best nitrogen source.

Table 9. Examination of Nitrogen Sources.

N source	Growth (OD550)	Total activity (units/ml)	Specific activity (units/mg)
Protein extract	9.8	3.95	2.93
Casamino acid	9.6	5.25	3.26
NZ amine	9.5	4.70	3.29
Tryptone	9.1	4.49	3.21
Yeast extract	8.0	3.27	3.21
Corn steep liquor	2.8	1.17	2.86
Bonito fillet extract	1.4		
Protein extract + yeast extract	11.5	6.80	4.05
NH4Cl + yeast extract	1.7		
Urea + yeast extract	0.2		

HB101 (pNT4553) was cultured in 350 ml of standard or altered medium A. Each N source was used as the protein extract at the concentration of 0.5%. Additional yeast extract with protein extract, NH 4 Cl and urea was used at the concentration of 0.1%. Total activity was expressed as units per ml of cultured broth. Specific activity was expressed as units per mg of protein.

Effects of aeration, and the concentrations of glycerol and protein extract

E. coli HB101(pNT4553) was cultured in standard medium A, whose glycerol and protein extract concentrations were altered, being used in the ratio of 3 to 1. In this case, the

ratio of the weight of total carbon and total nitrogen was about 10 to 1. I selected this ratio at first, because the amount of carbon was supposed to be sufficient for the materials for the biosynthesis and energy production. Fig. 17 shows the effects of the aeration conditions, which were altered by changing the volume of the medium in a shaking flask, and the concentrations of glycerol and protein extract on the DCase production of HB101(pNT4553). When the volume of the medium was large, aeration being estimated to be low, total activity and growth were optimum under the conditions of 4.5% glycerol and 1.5% protein extract, but specific activity was almost equal under the conditions of more than 3% glycerol and 1% protein extract. When the volume of the medium was small, aeration being estimated to be high, total activity and growth were higher and optimum at higher concentrations of glycerol and protein extract, the activity and OD at 550 nm reaching 14.1 units $\cdot ml^{-1}$ and 41.9 under the conditions of 6.5% glycerol and 2.2% protein extract, respectively. The specific activity was little lower than that with low aeration, and optimum at higher concentrations of glycerol and protein extract.

The effects of further high aeration and a high concentration of protein extract were also investigated under the conditions of 6.5% glycerol (Fig. 18). As in Fig. 17, the aeration conditions were altered by changing the volume of the medium. When the volume of the medium was small (20 ml and 50 ml of the medium), aeration being estimated to be high, total activity and growth were optimum under the condition of 3% protein extract. But in case of 100 ml medium, they decreased slightly under the condition of a higher concentration of protein extract. The specific activity did not change very much under the examined conditions. Under the conditions of 6.5% of glycerol, 3% of protein extract, and 20 ml of the medium, the activity and OD at 550 nm became maximum, being 22.9 units $\cdot ml^{-1}$ and 63.8, respectively. The dry cell weight was 17.1 mg $\cdot ml^{-1}$.

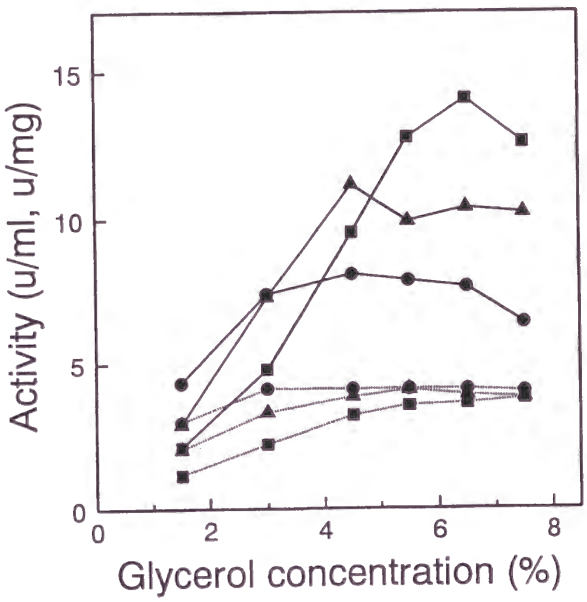

Fig. 17. Effects of the Concentrations of Glycerol and Protein Extract on DCase Production.

HB101(pNT4553) was cultured in 100 ml(■), 200 ml (▲), and 350 ml(●) of standard medium A, whose concentrations of glycerol and protein extract were altered, being used in the ratio of 3 to 1. Total activity, units·ml^{-1}-cultured broth, is shown by a solid line, and specific activity, units·mg^{-1}-protein, is shown by a dotted line.

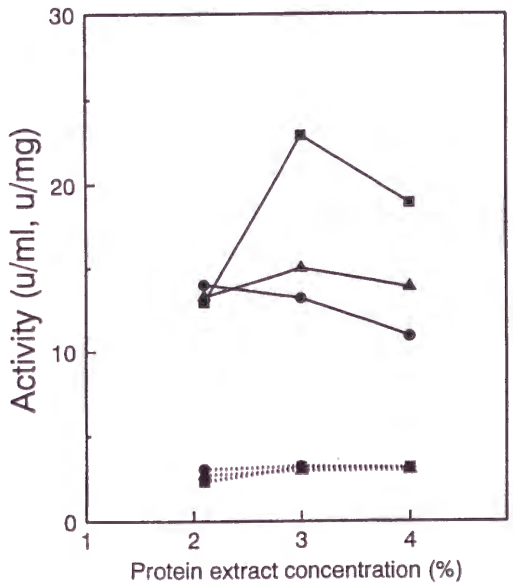

Fig. 18. Effect of the Concentration of Protein Extract on DCase Production.

HB101(pNT4553) was cultured in 20 ml(■), 50 ml(▲),and 100 ml(●) of standard medium A, whose concentration of glycerol was 6.5% and that of protein extract was 2.2, 3.0, or 4.0%. Total activity, units·ml^{-1}-cultured broth, is shown by a solid line, and specific activity, units·mg^{-1}-protein, is shown by a dotted line.

DISCUSSION

The production of D-amino acids using an immobilized DCase is advantageous, as described in the previous chapter. The economical and efficient production of an immobilized enzyme is thus one of the most important subjects. To achieve this, I attempted to produce a high amount of DCase inexpensively.

Plasmid pNT4553, comprising vector plasmid pUCNT, and a DNA fragment which contains an ORF for the DCase, i. e. has no sequence upstream and downstream of the ORF, was constructed for expression of DCase in *E. coli*. Without an inducer, a cell-free extract of recombinant *E. coli* JM109 containing pNT4553 showed high specific activity (2.3 units·mg^{-1}-protein). Inducers are expensive, therefore high production of a DCase without an inducer is important to obtain the enzyme economically. Overexpression of the DCase by recombinant *E. coli* JM109 carrying pAD108 was described in Chapter Ⅰ. Plasmid pAD108 contains a 1.8 Kbp *Sal* Ⅰ-*Eco*R Ⅰ fragment from *Agrobacterium* sp. strain KNK712 at the multicloning site of pUC19. The 1.8 Kbp fragment comprises a 912 bp open reading frame for the DCase, a 229 bp DNA upstream of the ORF, and a 646 bp DNA downstream of the ORF. In a cell-free extract of *E. coli* carrying pAD108, the DCase exhibited specific activity 3.3 units·mg^{-1}-protein with isopropyl-β-D-thiogalactopyranoside as an inducer (Chapter Ⅰ), and 2.2 units·mg^{-1}-protein without an inducer (unpublished results), which was almost the same activity as that of *E. coli* JM109 carrying pNT4553 without an inducer. As described in Chapter Ⅰ, even the recombinant *E. coli* carrying pAD108 produced a lot of DCase, about 50% of the soluble protein in a cell, so the expressed plasmid constructed in this study did not have much effect as to higher production of the enzyme compared to pAD108. However, plasmid pNT4553 was maintained more stably than pAD108, the former exhibiting 100% stability at the fourth cultivation, but the latter only 12% at the second cultivation (unpublished results). Plasmid pNT4553 seemed to undergo replication easily and to be stabilized when its excess sequence was reduced. The stable maintenance of the plasmid in the host cells is necessary for industrial scale up of the culture. Besides pNT4553 which had a

lac promoter, expression vector which had *trc* promoter was examined, but the DCase activity was almost the same as that with pNT4553 (data not shown).

The activity of the DCase and the stability of the plasmid depended on the type of *E. coli*, and HB101 was selected as the best host. *E. coli* HB101(pNT4553) showed the highest activity, and its plasmid was stably remained. The specific activity of the cell-free extract of *E. coli* HB101 (pNT4553) without an inducer was 3.66 units·mg^{-1}-protein, which was 10% higher than that of *E. coli* JM109 (pAD108) with an inducer, as described in Chapter Ⅰ. Also, the highly expressed DCase appeared not to form inclusion bodies. Thus, this strain has suitable characteristics for the economical industrial production of the DCase. All the *E. coli* strains examined here were derivatives of *E. coli* K-12, and so it is supposed that the genetic backgrounds were similar. But a little mutation in the chromosome seemed to affect the enzyme productivity, and the efficiency of replication and distribution of the plasmid.

The medium for the cultivation of HB101(pNT4553) was investigated to achieve economical production of a high amount of the DCase. Because of the effective assimilation of natural carbon and nitrogen sources, such as casamino acids and protein extract, which contain a lot of peptides, they were good for activity and growth. They also seemed to contain sufficient amounts of nutritional elements which are necessary for the host cells, that are auxotrophic mutants, and they may contain small quantities of other useful elements for growth and enzyme production. Glycerol was used as a carbon source together with other components. It was better for the growth and activity of the recombinant *E. coli* compared to other carbohydrates (unpublished results). When the recombinant *E. coli* was cultured under the condition of a smaller volume of the medium, aeration being estimated to be higher, the total activity and growth were higher, and were optimum at higher concentrations of glycerol and protein extract. Higher total activity was mainly caused by the increase of cell density. At maximum, the activity and dry cell weight reached 22.9 units·ml^{-1} and 17.1 mg·ml^{-1}, respectively. This activity was about 4 times higher than that under the cultivation conditions in 2YT medium, and about 26 times higher than that of *Agrobacterium* sp. strain KNK712. It is supposed that carbon and nitrogen sources are utilized more effectively under high aeration

conditions, and so the materials and energy seem to be sufficient for growth and enzyme synthesis. But with high concentrations of these medium components, growth was inhibited slightly. Therefore, higher aeration, and divided feeding of carbon and nitrogen sources will enable high growth and high production of the enzyme.

I achieved the economical hyperproduction of the thermotolerant DCase by constructing a superior host-vector system and by optimizing the culture conditions with only cheap medium components. Using the highly produced DCase, the immobilized enzyme could be prepared advantageously. Such studies on DCase, which is industrially an important enzyme, have not yet been reported, and so they are significant for improvement of the production of D-amino acids. Further study on the effective production of DCase, such as cultivation in a jar fermentor and other host vector systems, should be performed.

SUMMARY

For the high level production of the thermotolerant DCase in the recombinant *E. coli*, a plasmid pNT4553 was constructed. The amount of the DCase activity and the stable existence of the plasmid in the host cells were dependent on the *E. coli* strains. *E. coli* HB101 was the best host strain among 13 types of *E. coli* tested. *E. coli* HB101 expressed the highest activity, i.e. 6.36 units·ml^{-1}-cultured broth in 2YT medium (1.6% of tryptone, 1.0% of yeast extract, and 0.5% of NaCl, pH7.0). And the plasmid was stably maintained on cultivation in the cases of 5 types of E. coli including HB101.

Casamino acids, NZ-amine, peptone, and a mixture of hydrolyzates of corn gluten, wheat gluten and soybean (protein extract), were good for both activity and growth as natural nitrogen sources. When cultivation was carried out under the conditions of high concentrations of glycerol (6.5%) as a carbon source, and protein extract (3.0%) as a nitrogen source, and small volume of the medium (20 ml of medium in a 500 ml of shaking flask), aeration being estimated to be high, growth and activity reached OD550 = 63.8 (17.1 mg-dry cell weight·ml^{-1}-cultured broth) and 22.9 units·ml^{-1}-cultured broth, respectively. The

economical hyperproduction of the DCase using only inexpensive medium components was achieved.

CONCLUSION

In this thesis, I reported that high-active DCase was obtained and was highly produced economically. I also described that the DCase with improved thermostability could be used as an immobilized enzyme. The results described in each chapter can be summarized as follows:

Agrobacterium sp. strain KNK712, which produced DCase, was isolated from soil. The enzyme could catalyze the hydrolysis of several *N*-carbamyl-D-amino acids, and exhibit strict D-form stereoselectivity. Both the optimum pH and the pH that gave the greatest stability were about pH 7.0, the enzyme was stable at 50 ℃, and it was inhibited by thiol reagents. The DCase gene was cloned into *E. coli*, and was overexpressed as an active form under the control of the *lac* promoter with an inducer, DCase accounting for 50% of the soluble protein in the cells. Thus, an appropriate DCase for the production of D-amino acids was obtained, and was highly produced with genetically engineered *E. coli* (Chapter Ⅰ).

The DCase produced with *E. coli* was immobilized. The porous polymers, Duolite A-568 and Chitopearl 3003, were much better than other resins as to the activity and stability of the adsorbed enzyme. DCase immobilized on Duolite A-568 was found to be most stable at about pH7, and it was further stabilized by reductants such as dithiothreitol, cysteine, cysteamine and sodium hydrosulfite. The higher crosslinking degree with glutaraldehyde also stabilized the immobilized enzyme during reactions. Thus, excellent immobilization methods and usage of the immobilized enzyme stably were found. Furthermore, the superior stability of the immobilized DCases of thermotolerant bacteria during the reaction suggested that the thermostability of the enzyme affected the stability during the reaction (Chapter Ⅱ).

DCase, in which amino acid residues were substituted by mutation, followed by selection based on thermotolerance, showed improved thermostability. The thermotolerant DCases immobilized on Duolite A-568 showed higher activity than the immobilized native DCase. From the results of repeated batch reactions, the half-lives of the activities of immobilized thermotolerant DCase, in which Leu was substituted for Pro 203, and

immobilized native DCase were 104 and 58 times, respectively. It was revealed that the higher thermotoleance enabled the immobilized enzymes to be more stable in reactions. Compared with soluble DCase, immobilized DCase was somewhat stable, and its activity was optimum at a lower pH. Using the improved DCase, the activity and stability of the immobilized enzyme in the reaction should be sufficient for practical use (Chapter Ⅲ).

For the economical hyperproduction of thermotolerant DCase in *E. coli*, recombinant plasmid pNT4553 was constructed, which was stably maintained in the host cells. *E. coli* HB101, in which the activity of the DCase and the stability of the plasmid were the highest, was selected as the best host. Without an inducer, the specific activity of a cell-free extract was more than 10 times higher than that of *Agrobacterium* sp. KNK712. When cultivation was carried out (1 under the conditions of high concentrations of glycerol, as a carbon source, and protein extract, as a nitrogen source, and high aeration conditions, the growth and activity reached OD550 = 63.6 7.1 mg·ml^{-1}-cultured broth) and 22.9 units·ml^{-1}-cultured broth, respectively. The total activity (units·ml^{-1}) was about 26 times higher than that of *Agrobacterium* sp. KNK712 with only cheap medium components. Using the highly produced DCase with inexpensive materials, the immobilized DCase could be obtained at a low cost, and so the practical use of the immobilized DCase was possible (Chapter Ⅳ).

As described above, the thermotolerant DCase could be used as a bioreactor. This study produced a new biotransformation process for actual D-amino acids production.

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